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Characterization of micro-components of avocado oil extracted with supercritical carbon dioxide and their effect on its oxidative stability

by

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Submitted in partial fulfillment of the requirements for the degree of

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DECLARATION

I declare that the thesis which I herewith submit at the University of Pretoria for the award of the degree of PhD (Food Science) is my research and has not been submitted by me for a degree to any other University or institution of higher education.

Mathilda Elizabeth Mostert
August 2007

Our life is an apprenticeship to the truth that around every circle another can be drawn; that there is no end in nature, but every end is a beginning, and under every deep a lower deep opens.

RALPH WALDO EMERSON

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ABSTRACT

Characterization of micro-components of avocado oil extracted with supercritical carbon dioxide and their effect on its oxidative stability

By

Mathilda Elizabeth Mostert

Promotor: Dr K.G. Duodu
Co-promoters: Dr L. M. du Plessis
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Department: Food Science
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The main objective of this study was to determine the effect of fruit ripeness and drying method on the oxidative stability and micro-component content of avocado oil extracted with supercritical carbon dioxide (SC-CO_2). A secondary objective was to determine the effect of fruit ripeness, method of fruit drying and extraction method on the extractability of avocado oil with hexane and SC-CO_2.

For the oil extractability study, unripe and ripe avocado fruit pieces were either freeze-dried or oven-dried (80^{o}C) and extracted with hexane or SC-CO_2. For both extraction methods, oil yield was higher from ripe fruit than from unripe fruit. Scanning electron microscopy (SEM) indicated structural degradation during ripening, making the oil more available for extraction in ripe fruit. Oil from freeze-dried samples was in most cases more extractable than from oven-dried samples possibly through formation of rigid structures due to starch gelatinisation and dehydration and protein crosslinking around the oil cells during oven drying. Oil yield was higher with hexane than with SC-CO_2 extraction because hexane is less selective, permeates the whole plant material and leads to a more complete extraction, while SC-CO_2 may create paths of least resistance in the plant material where it moves preferentially, thus leading to a less complete extraction.

For oxidative stability studies and micro-component characterisation, oil extractions were performed on an industrial scale SC-CO_2 extractor. For all treatments (unripe freeze-dried, ripe freeze-dried, unripe oven-dried, ripe oven-dried), oil was divided into four fractions and analysed for fatty acid profile, peroxide value (PV), anisidine value (AV), free fatty acids (FFA), oxidative stability index (OSI), colour, tocopherol, sterol, chlorophyll, carotenoid and total unsaponifiable content.

Oil from ripe, freeze-dried avocado had relatively lower levels of chlorophyll, carotenoids and tocopherols, than oil samples from the other treatments. This may be due to relatively higher lipoxygenase levels in ripe fruit which may bring about higher oxidative breakdown of these components. Also, the activity of lipoxygenase may be preserved under the lower temperature conditions of freeze-drying, but inactivated at high temperature during oven-drying.

Intensity of blue and red on the Lovibond colour scale of all oil samples as well as chlorophyll and carotenoid content increased with progressive extraction. These pigments are presumably extracted in the latter stages of extraction because they are located in chloroplasts, chromoplasts and idioblast cells with thicker membranes than the parenchyma cells where triglycerides are located. Levels of total sterols, total tocopherols and their isomers did not show any specific trends with progressive extraction, which could be related to their location in cell membranes where they would be extracted concurrently with the triglycerides. Levels of total unsaponifiables were mostly higher in the first than the latter fractions. This could be due to the early elution of non-polar waxes which are highly soluble in SC-CO_2 and highly available due to their location on the surface of the avocado skin.

The fatty acid profile of the avocado oil was not influenced by the degree of ripeness or drying method and therefore did not affect the OSI. Oleic acid increased while linoleic acid decreased with progressive extraction. Compared to the changes observed in levels of some of the micro-components, the changes in fatty acid levels with progressive extraction were

relatively small and the fatty acid profile alone could not explain the OSI of the oil. Oil from oven-dried avocado had lower PVs but higher AVs than oil from freeze-dried fruit indicating more advanced oxidative deterioration in oil from oven-dried samples than from freeze-dried samples. FFA levels were higher in oil from ripe, freeze-dried fruit. Levels of hydrolytic enzymes increase during fruit ripening and are preserved during freeze-drying while they are inactivated during oven-drying. FFA levels decreased with progressive extraction. Free fatty acids are very soluble in the SC-CO_2 and due to their location on the surface of the plant material, they could be extracted early in the extraction.

Oil from oven-dried fruit had relatively higher OSI compared to the other treatments. The OSI of all samples increased with progressive extraction. There was a significant negative correlation between FFA and OSI for both drying methods. AV correlated positively with OSI for oil from oven-dried fruit and negatively for oil from freeze-dried fruit. AV contributed the most to the prediction of OSI in oven-dried fruit, while FFA contributed the most in freeze-dried fruit. It was suggested that the high OSI of oil from oven-dried fruit, despite its high AV, may be due to the presence of compounds with high antioxidant activity in the oil formed through the high temperatures of the oven-drying process. Therefore, using multiple regression techniques, predictive models were developed to determine the effect of the micro-components on the oxidative stability of the oil.

The OSI correlated positively with chlorophyll (0.83) and carotenoids (0.80). The models indicated that chlorophyll and carotenoids were the most important variables in predicting the oxidative stability of avocado oil extracted with SC-CO_2. This might be due to the antioxidant effect of carotenoids and the possible formation of pheophytin and pyropheophytin, thermal breakdown products of chlorophyll, which exert antioxidant effects in oil.

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1. Introduction and Literature Review

1.1 Statement of the problem

The avocado (*Persea americana Mill.*) belongs to the Lauraceae family and is placed in one of three groups or races for horticultural purposes: *americana* (West Indian), *guatemalensis* (Guatemalan) and *drymifolia* (Mexican) (Sippel, 2001). South Africa produces 34 000 tons of avocado fruit per year (Mr. Derek Donkin, 2004, South African Avocado Growers' Association, personal communication) of which the main cultivars are 'Fuerte' (Guatemalan/Mexican hybrid) and 'Hass' (Guatemalan), which make up 38% and 36% of the area planted to avocados respectively. Of this fruit 13 800 tons are used for the production of oil in local oil factories. When seasonal variation is taken into account, these avocado oil-producing factories have an average yield of 10% oil per ton fruit utilised (Mr. Dennis Gilbert, 2003, Specialised Oil, Personal Communication; Mr. Daan Jacobs, 2003, Hans Merensky Oils, Personal Communication).

The two main processes used for the production of avocado oil include a centrifugal extraction process and a cold press extraction of heat-dried avocado fruit. Ripe fruit is used for the centrifugal process, whilst unripe fruit is used for the cold press extraction method. It is a recognized fact that the unsaponifiable fraction of avocado oil is rich in phytochemicals, including polyphenols, sterols and tocopherols (Farines, Soulier, Rancurel, Montaudoin & Leborgne, 1995; Eyres, Sherpa & Hendriks 2001). However, neither of these two processes produces a product containing significant amounts of antioxidants (Prof. Ben Botha, 2003, Tshwane University of Technology, personal communication). Factors influencing the breakdown of these compounds might include heat drying, which is used as a pre-treatment before avocado oil extraction with the cold press method. The ripening stage of the fruit may also play a significant role.

A few studies have been undertaken to distinguish between compositional differences during the maturation stages of the avocado, mainly the fatty acid profile (Lozano, Dhuique Mayer, Bannon & Gaydou, 1993; Poiana, Giuffre & Mincione;

1999). Like all climacteric fruit, avocado only starts to ripen after harvesting, which takes place at horticultural maturity (Awad & Lewis, 1980; Sippel, 2001; Ozdemir & Topuz, 2004). The carotenoid and chlorophyll content seems to decrease during ripening (Ashton, Wong, McGhie, Vather, Wang, Requejo-Jackman, Ramankutty, & Woolf, 2006). Heat drying of the unripe fruit, on the other hand, might lead to the degradation of tocopherols, carotenoids and polyphenols. Preservation of these antioxidants in avocado oil as an edible oil is important, not only because they influence the oxidative stability of the oil, but also for their function in the prevention of ailments like cardiovascular disease caused by atherosclerosis (Ohr, 2002).

Other extraction methods for avocado oil include solvent extraction using organic solvents like hexane and supercritical fluid extraction with carbon dioxide. Although hexane extraction is a mild, well-known extraction method, large amounts of solvent is needed which is expensive and environmentally hazardous. Carbon dioxide is a non-toxic and environmentally compatible fluid for the extraction of edible oils (Garcia, Lucas, Rincon, Alvarez, Gracia & Garcia 1996; King, 1997). Supercritical carbon dioxide (SC-CO_2) extraction has been proven to be a viable alternative for hexane as avocado oil extracted with these two methods have been shown to have similar fatty acid profiles (Botha & McCrindle, 2003). The micro-component content and composition as well as oxidative stability of avocado oil extracted with SC-CO_2 have, however, not been determined. Furthermore, the effects of progressive extraction on the micro-component distribution and oxidative stability is not known.

South Africa has a large agricultural sector and there is increasing interest in the growth of small and developing farmers. If a new market for avocado fruit can be created by production of high quality avocado oil at a premium price, more small and developing farmers would be interested in cultivating avocado fruit. This will in turn benefit the agricultural and economic sectors in the long term. Oil production can also create an alternative market for the commercial farmer, which has the benefit of less risk, compared to the fresh fruit market where visual appearance of the fruit is very important.

1.2 Literature review

1.2.1 Avocado fruit and avocado oil

Although known to the natives of tropical America, the avocado was described for the first time in 1499 when it was observed growing in a small harbour at the foot of the Sierra Nevada de Santa Marta, USA (Sippel, 2001). Major avocado growing areas in the world include Mexico, the USA, Brazil, Israel, Chile, South Africa, Spain and Australia (Knight, 2002). Little has been recorded about early introductions of avocado into South Africa, but it is accepted that the first trees were West Indian race seedlings planted on the coastal strip of KwaZulu Natal, especially around Durban, in the late 19th century. Avocados are now widely grown in South Africa, principally in the Limpopo Province and Mpumalanga and to a lesser extent in KwaZulu Natal. Trees of Mexican and Guatemalan origin proved to be better adapted to South African climatic conditions. Production in South Africa is dominated by two cultivars of the genus and species *Persea americana* Mill, namely *Fuerte* and *Hass*.

The avocado fruit has been accredited with several health claims (Eyres *et al*., 2001). One of these claims include the lowering of total cholesterol and low-density lipoprotein levels, without changing high-density lipoprotein levels, when included in the diet (Colquhoun, Moores, Somerset, & Humphries, 1992).

Avocado is one of the few cultivated fruits in which oil is a main component on dry basis (Werman & Neeman, 1987). The oil content is in the range of 15-30% depending on the variety, and is mainly mono-unsaturated with the predominant fatty acid being oleic acid (Werman & Neeman, 1987). According to Werman and Neeman (1987), of all fruits only olive and palm can rival the avocado in oil content.

Avocado oil is valued as an edible oil due to its health-enhancing qualities and is especially used in the treatment of connective tissue diseases (Maheu, Le Loet & Loyau, 1995). This oil is of good quality because the processed fruit from which the oil is obtained is still intrinsically sound and is only termed second grade because of

its appearance (black or brown spots, rough skin, shape and size), which is not appealing to the consumer (Eyres *et al*., 2001).

Avocado cultivars produced in South Africa and their oil contents are listed in Table 1.1.

Table 1.1: Oil content and harvesting time of Avocado cultivars produced in South Africa (Kaiser, Keevil, Levin & Wolstenholme, 1996)

Cultivar	Oil content (% wet basis)	Harvesting time (warm climates)
Fuerte	26 – 40	June – September
Hass	± 25	June – October
Edranol	Max 20	June – September
Pinkerton	± 20	June
Nabal	10 – 15	October – November
Ettinger	± 24	March – June
Bacon	Max. 22	April – June
Alboyce	Max. 22	March – August
Ferdyn	± 22	March – July
Sharwill	± 22	April – June
Teague	± 10	February – May
Santana	± 12	April – May
Wurtz	Max. 21	July – September

1.2.2 Morphology of avocado fruit

The avocado fruit consists of a skin, mesocarp (which is divided into a green part near the skin and a yellow part near the stone) and a large stone (Figure 1.1) (Somogyi, Barrett & Hui, 1996). The mesocarp of the avocado fruit has a fairly uniform cellular composition, consisting primarily of large parenchyma cells and idioblast cells (which comprise 2% of the mature fruit volume). Parenchyma cells

differ in two major ways from idioblast cells, namely their structure and their contents.

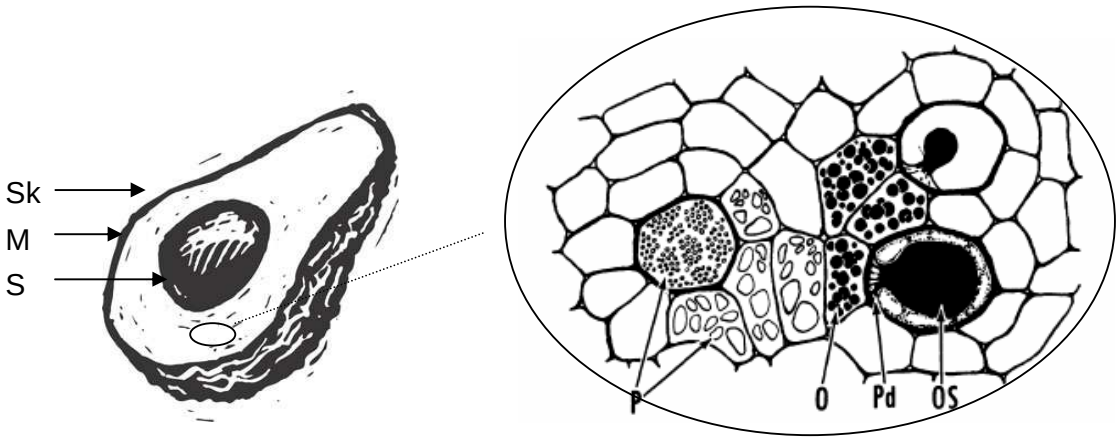


Figure 1.1: Diagram of avocado fruit morphology and avocado mesocarp cells where Sk, Skin; M, Mesocarp; S, Stone; P, pits; O, oil; OS, oil sac in idioblast cell; Pd, plasmodesmata (Scott, Bystom & Bowler, 1963)

Parenchyma cells only have thin, primary walls (Mauseth, 1995). The idioblast cells on the other hand, are surrounded by a specialised cell wall composed of the primary cellulosic wall, a secondary suberin layer and a tertiary wall (Platt-Aloia, Oross & Thomson, 1983). During ripening, which only occurs after the fruit is picked, the primary walls of the parenchyma cells are degraded due to the activities of the cell wall hydrolytic enzymes, namely cellulase and polygalacturonase and fruit softening occurs. The suberised wall of the idioblast oil cells is, however, immune to the activity of these enzymes and remains intact during ripening (Platt & Thomson, 1992).

Parenchyma cells mainly contain numerous droplets of lipid substances, mostly triacylglycerol (Platt & Thomson, 1992). The oil in the idioblast cells occur as a single large drop filling the cell, has a different appearance in freeze fracture replicas compared with the triacylglycerols and is therefore thought to have a different

composition (Platt-Aloia *et al*., 1983). Scott *et al*. (1963) noted a difference in staining between the content of the parenchyma and idioblast cells and attributed this to possible traces of terpene compounds present in the oil sacs of the idioblast cells. Werman and Neeman (1987), however, claim that most of the oil in avocado fruit is located in the idioblast cells and that only small droplets of oil can be detected in the parenchyma cells. Compounds isolated from the idioblast oil cells include those with anti-fungal, antifeedant and insecticidal activity (Prusky, Plumbley & Kobiler, 1991; Rodriguez-Saona, Millar & Trumble, 1997; Rodriguez-Saona, Millar & Trumble, 1998[a]; Rodriguez-Saona, Millar, Maynard, & Trumble 1998[b]). These effects have been attributed to the presence of unique compounds present in the idioblast cells including persin ((12Z,15Z)-1-acetoxy-2-hydroxy-4-oxo-heneicoxa-12,15-diene) and several 2-alkylfurans (Rodriguez-Saona *et al*., 1998[a]).

1.2.3 Avocado oil production

The first step in production of avocado oil involves extraction of the oil from the fruit using methods including solvent extraction, mechanical pressing, centrifugation of pulp slurries and enzymatic extraction using a mixture of polygalacturonases, α-amylase and a protease. (Bizimana *et al*., 1993; Buenrostro & López-Munguia, 1986). Hard, mature, fruit are used for solvent extraction and mechanical extraction, while soft, mature seeded fruit are used for oil separation by centrifugation (Werman & Neeman, 1987).

Current methods used for extraction of avocado oil in South Africa include cold pressing of heat dried unripe fruit and the centrifugal extraction of ripe fruit (Mr. Dennis Gilbert, 2003, Specialised Oil, Personal Communication; Mr. Daan Jacobs, 2003, Hans Merensky, Personal Communication). However the application of heat in these methods could affect the functional properties of the micro-components in avocado oil negatively.

After extraction, the crude avocado oil undergoes refinement, bleaching and deodorisation to yield an edible oil. Similar to other well-known edible oils, avocado

oil is sensitive to oxidative processes resulting in rancidity, production of undesirable flavours and quality losses during storage (Werman & Neeman, 1986).

1.2.4 Methods of extraction of avocado oil

1.2.4.1 Mechanical pressing

In general, mechanical extraction of vegetable oils includes two methods namely hydraulic expelling and screw pressing. Mechanical pressing is usually used for materials exceeding an oil content of 20%, while solvent extraction is recommended for products like soybeans or press cakes having an oil content of less than 20% (Carr, 1997).

Screw presses are used in higher technology areas throughout the world for expulsion of oil from copra, palm kernel, peanut, cottonseed and flax seed amongst others (Carr, 1997). Avocado oil has also been successfully expelled from sun-dried, destoned avocado fruit using a screw press (Southwell, Harris & Swetman, 1990). Due to the high water content of avocados, pressing of the raw flesh is problematic, and fruit is normally air dried prior to screw pressing (Southwell, Harris & Swetman, 1990). The effect of the air drying on the oxidative stability of the oil is not clear. In principle, a screw press is a continuous screw auger designed to accept feed and subject it to gradually increasing pressure as it is conveyed through a barred cage. Disrupted or distorted oil cells act as capillaries which are reduced in volume as pressure is applied and the oil is expelled (Ward, 1976).

Hydraulic pressing uses the principle of gradually increasing pressure on the incoming material as it progresses through the interior of a closed barrel. Oil extracted in this manner is traditionally called “cold pressed” oil (Carr, 1997). Hydraulic pressing of avocado fruit has been well documented and implemented for the extraction of avocado oil for several years (Love, 1944). Avocado oil recovery from mechanical pressing varies between 79.4 – 90.3 % (Southwell, Harris & Swetman, 1990).

1.2.4.2 Solvent extraction

Together with mechanical extraction using centrifugal force, solvent extraction was probably, until recently the most common method of extracting oil from avocado fruit (Southwell, Harris & Swetman, 1990). Hexane has become the solvent of choice for solvent extraction because of high stability of the solvent, low evaporation loss, low corrosiveness, little greasy residue and better odour and flavour of the extracted products (Johnson, 1997).

Solvent extraction has several drawbacks including high capital equipment cost and operational expenditures, the perpetual hazard of fire and/or explosion as well as the residual solvents associated with both the oil and the meal including endocrine-disrupting compounds like phthalates that can lead to the production of androgens in the body (Owusu-Ansah, 1997; Petrovic, Eljarrat, Lopez de Alda & Barceló, 2004).

The primary prerequisite for solvent extraction for oils is the rupturing of the seed or feed material to render the cell wall more porous. According to Diosady, Rubin, Ting and Trass (1983), complete rupturing of the cell wall is necessary for rapid solvent extraction. In a study conducted by Ortiz, Dorantes, Gallndez and Cárdenas (2004), the shape of the idioblast cells of avocado fruit became irregular and rough-shaped after hexane extraction. According to Ortiz *et al*. (2004), hexane extraction of an unknown avocado cultivar yielded approximately 59% oil from the avocado pulp. An avocado oil yield of 74-75% (dry basis) from the *Fuerte* variety has been obtained using petroleum ether for an extraction time of four hours (Lewis, Morris & O'Brien, 1978).

1.2.4.3 Centrifugation of pulp slurries

Centrifugation of pulp slurries is mostly used in the olive oil industry and is used in South Africa for the extraction of avocado oil from ripe fruit (Mr. Dennis Gilbert, 2003, Specialised Oil, Personal Communication; Mr. Daan Jacobs, 2003, Hans Merensky Oils, Personal Communication). This process is also lately referred to as "cold pressing" (Eyres *et al*., 2001). The olive or avocado fruit is first put through a hammer mill and the paste is pumped to a malaxeur where it is warmed and beaten

or mixed until the oil begins to separate (Benedito, Mulet, Clemente & García-Perez, 2004). The paste is then pumped to a centrifuge where the solids are separated from the liquids. In some instances water is added and this process is also referred to as the "aqueous extraction process" (Cater, Rhee, Hagenmaier & Mattil, 1974). The vegetable water and oil are further separated in a final centrifugal process. The yield obtained from this method is generally high, it requires limited labour and is continuous and automated (Ranalli & Martinelli, 1995). It is however expensive, has a high energy consumption, yields a varying amount of vegetable water to be disposed of and has reduced antioxidant levels due to added water.

Modern centrifugal olive oil processing units have been modified to suit the parameters of the avocado fruit (Eyres *et al*., 2001). The adaption of certain parameters and the addition of chemicals have been introduced to optimize oil extraction from the avocado fruit. The effects of centrifugation rate, pH and sodium chloride on extraction yield were extensively studied by Werman and Neeman, (1987). The addition of inorganic salts like $CaCO_3$ and $CaSO_4$ has also been proven to increase oil yield (Bizimana *et al*., 1993). There was no indication in any studies of how these parameters affected oxidative stability.

1.2.4.4 Enzymatic extraction

Enzymes are generally considered environmentally friendly and the utilization of enzymes for oil extraction with regards to increasing yields and reducing side products have long been recognised. They are probably the most efficient way to rupture cell walls, even at molecular level (Fullbrook, 1983) and can be synergistically used with other solvents or physical means to extract oils and fats from plant material (Owusu-Ansah, 1997). Due to the structural complexity of plant material, the extent of enzymic degradation of the cell wall is determined by the structural details, such as the chemical constituents and the type of source of the enzymes. Some of the most widely used enzyme actions used in extraction of vegetable oils include protease, cellulase, polygalacturonase and amylase activity (Owusu-Ansah, 1997). Enzymes used in the extraction of avocado oil include α-

amylase and a mixture of protease and cellulase (Buensrostro & López-Munguía, 1986).

Enzymic extraction of fat can be divided into three categories namely: Enzyme-Enhanced Solvent Extraction, Enzyme-Assisted Expelling and Enzyme Assisted Aqueous Extraction. In all of these, the objective of using enzymes is to break the cell wall and release the oil by some mechanical means (Owusu-Ansah, 1997).

Enzyme aided aqueous extractions that have been carried out include those on coconut (McGlone, Canales & Carter, 1986), melon seed (Fullbrook, 1983) and avocado (Buenrostro & Lopez-Munguia, 1986). It seems that the enzyme assisted extraction process is more conducive for materials with higher oil-to-protein ratios. Satisfactory yields have, for instance, not been obtained for soybeans unless excessive hydrolysis of the proteins is effected (Owusu-Ansah, 1997).

The extraction of oil from fruits like avocado is enhanced by partially accelerating the natural enzymatic breakdown processes within the avocado paste, so favouring the separation of oil from other macromolecules to which oil is linked (Domínguez, Núñez & Lema, 1994). Although olives are the most studied of the oil fruits, avocado oil extraction studies on laboratory scale have indicated an increase of 4-5 times superior to olive oil after enzymatic treatment. α- Amylase proved to be the best enzyme for the optimization of oil extraction from avocados (Domínguez *et al*., 1994).

1.2.4.5 Supercritical fluid extraction

Supercritical fluid extraction is a unitary mass transfer operation based on the use of fluids at temperatures and pressures above the critical values. These conditions make the supercritical fluid present peculiar physicochemical properties between the gas and liquid states, which confer them with exceptional solvent characteristics (Gómez & De la Ossa, 2002). Supercritical fluid extraction (SFE) has been used since the early eighties when extensive work was performed in industrial research laboratories (Manigold, 1983). Supercritical fluid extraction is an alternative to

traditional separation processes, which is used when the separation of thermally labile substances and attainment of high-purity products is the target (Araujo *et al*., 2001). The technology of supercritical fluid extraction has been applied to fragrances, cosmetics, food and various chemical industries (Palmer & Ting, 1995; Garcia *et al*., 1996; Turkay, Burford & Sangum, 1996).

Although supercritical fluids like methylene chloride (CH_2Cl_2) have been used in the past for the removal of caffeine from coffee, it is no longer used because it is hazardous in the workplace and difficult to remove completely from the coffee (Kotz & Treichel, 1999). Currently, supercritical carbon dioxide (SC-CO_2) is finding more application in the food industry; the best known application probably being the decaffeination of coffee (Kotz & Treichel, 1999). SC-CO_2 has also been used for the enrichment of oil in micro-components to obtain products with improved functionality for specific applications or with better nutritional values (Ibáñez, Benavides, Señoráns, & Reglero, 2002). Co-solvents used for the SC-CO_2 extraction of lipids from plant material include isopropanol and methanol (Kuk & Hron, 1994).

The principle of supercritical fluid extraction can be explained by using a phase diagram for carbon dioxide (Figure 1.2): The boiling point of carbon dioxide at 1 bar is -78.5ºC, its critical pressure is 72.9 bar and its critical temperature is 31.1ºC (Manigold, 1983).

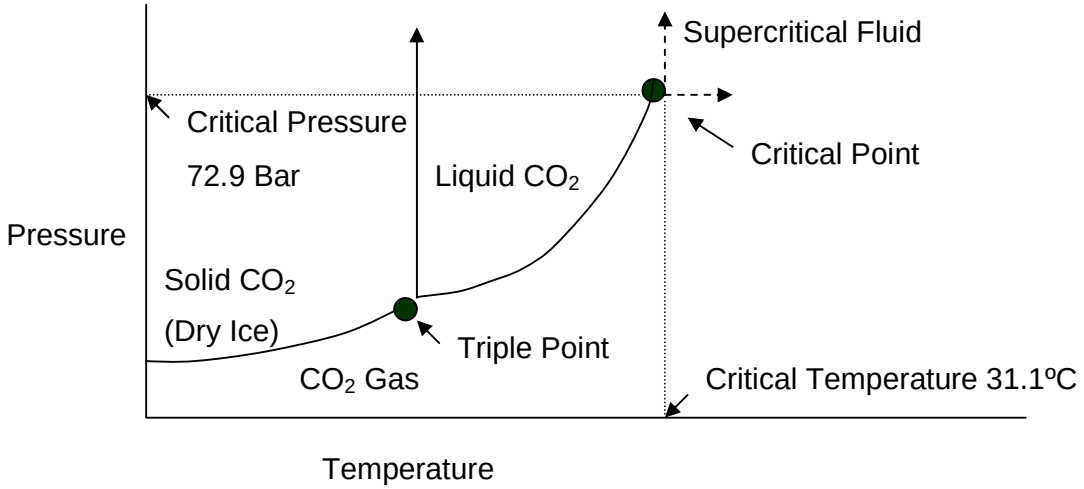


Figure 1.2: Phase diagram for carbon dioxide (King, 1997).

A phase of a substance is a form of matter that is uniform throughout in chemical composition and physical state and matter is usually referred to in its gaseous, liquid or solid state (Atkins, 1994). A spontaneous phase transition occurs at a characteristic temperature for a given pressure. The phase diagram shows the regions of pressure and temperature at which various phases are thermodynamically stable. The boundaries between phases show the values at which two phases coexist in equilibrium.

When pressure is applied to a heated liquid in a sealed vessel, the conditions differ from that of liquid in an open vessel. When liquid is heated in a sealed vessel, boiling does not occur. Instead, the temperature, vapour pressure and the density of the vapour rise continuously. Simultaneously, the density of the liquid decreases as a result of its expansion. When the density of the vapour is equal to that of the remaining liquid, the surface between the two phases disappears. The temperature and pressure at this point (the critical point) is called the critical temperature and critical pressure (shown in Figure 1.2 for CO_2). At and above this temperature a single phase exists and an interface no longer exists (Araujo *et al*., 2001).

Above the critical point, the liquid and gaseous phases become identical and indistinguishable. This phase has the high density of a liquid, but the low viscosity of a gas and is known as a supercritical fluid (Petrucci & Harwood, 1997). Molecules in supercritical fluids are in much closer proximity than in ordinary gases and exert strong attractive forces on the molecules of a liquid or solid solute. Therefore, both liquids and solids become much more soluble in a gas above its critical pressure and temperature i.e. in the supercritical region (King, 1997).

The main advantage of supercritical fluids over liquid solvents is that their high diffusivity, low viscosity and low surface tension can speed up mass transfer-limited extractions (Garcia *et al*., 1996). A single supercritical fluid may also substitute for a variety of liquid solvents because it offers the possibility of modifying product solubilities through altering the pressure and/or temperature (Garcia *et al*., 1996). In

general, the solubility of a substance in supercritical fluids increases with pressure when kept at a constant temperature (Garcia *et al*., 1996).

The procedure used in the extraction of natural products with SC-CO_2 is simple. The carbon dioxide is condensed in a diaphragm compressor to a pressure in excess of its critical pressure and a temperature in excess of its critical temperature. The fluid or SC-CO_2 flows through an extraction vessel containing the plant material. By lowering the pressure in two stages, below the critical pressure of carbon dioxide, the extracted oil is recovered from its solution (Manigold, 1983). It is also possible to isolate the oil by decreasing the pressure and increasing the temperature simultaneously. The released gas is condensed and recompressed, thus completing the cycle (Manigold, 1983).

According to Gómez and de la Ossa (2002), the yield of seed oil obtained with SC-CO_2 increases with increasing operating pressure. A higher flow rate also led to a somewhat higher yield, but with a much higher solvent (SC-CO_2) consumption. A lower flow rate, on the other hand, reduced solvent consumption but produced notably lower yields. An increase in particle size of the seed to be extracted is associated with a decrease in extraction yield due to the increase of the mass transfer resistance between the surface of the seed and the SC-CO_2 (Gómez, López & de la Ossa, 1996).

It appears the only work done on SC-CO_2 extraction of avocado oil was that reported by Botha and McCrindle (2003). The optimum extraction pressure was determined to be 540 atmosphere (atm), yielding a 95% complete extraction after one hour. A 94% extraction was complete within two hours when the pressure was reduced to 350 atm. A substantial decrease in capital investment was achieved when the pressure was reduced, even though this resulted in a longer extraction period (Botha & McCrindle, 2003). They also proposed supercritical extraction method as an alternative for hexane extraction of avocado oil as no significant difference in fatty acid profile was reported.

1.2.4.6 Yield and composition of plant oils extracted with SC-CO_2 compared with hexane extracts

The apparent yield from SC-CO_2 extraction of seed oil is slightly lower than extraction with hexane (Friederich & List, 1982; Gómez & de la Ossa, 2002; Bravi, Spinoglio, Verdone, Adami, Aliboni, D'Andrea, De Santis & Ferri, 2005). This has been attributed to the fact that as an extraction solvent, hexane is much less selective than SC-CO_2 (Gómez & de la Ossa, 2002) and is able to extract a wider variety of compounds such as phospholipids, waxes and pigments, thus contributing to higher oil yield. This selectivity is further demonstrated by the lower acidity index of oils extracted with SC-CO_2 (Bernardo-Gil, Grenha, Santos, & Cardoso, 2002). A darker green oil, possibly related to the higher chlorophyll content was reported for avocado oil extracted with hexane when compared to SC-CO_2. (Botha & McCrindle, 2003). Bhattacharjee, Singhal and Tiwari (2007) also reported increased pigmentation with the average gossypol content of hexane-extracted cotton oil being 0.242% while that of SC-CO_2-extracted cotton oil was 0.015%.

The amount and composition of triglycerides in oils extracted with SC-CO_2 and hexane is very similar (Botha & McCrindle, 2003). Significant differences have been observed for the unsaponifiable fraction of oils extracted with these two methods (Gómez *et al*., 1996; Gómez & de la Ossa, 2002). More unsaponifiables were obtained with hexane extraction which is probably also due to the lower selectivity of hexane. Oil extracted with SC-CO_2 shows significantly less phosphorus and correspondingly less chromatographic refining loss than hexane-extracted oil (Friederich & List, 1982). Due to the insoluble nature of phospholipids in SC-CO_2, oil extracted with SC-CO_2 is normally low in phospholipids, resulting in a lower oxidative stability than that of oils extracted with conventional solvent or screw press methods (List & Friedrich, 1989). The SC-CO_2 extracted oil, therefore, has the advantage of being essentially equivalent to a degummed, hexane-extracted crude oil.

1.2.4.7 Enrichment of oils with micro-components during SC-CO_2 extraction

SC-CO_2 can be used in the extraction of oils to produce oil fractions enriched in certain micro-components. Przybylski, Lee and Kim (1998) reported high colour measurements (values of tristimulus yellow, red and blue) and the presence of chlorophyll pigments in the last fractions of canola oil extracted with SC-CO_2. This suggests that during SC-CO_2 extraction of oil from plant material, pigments tend to be extracted towards the end of the process which results in the latter oil fractions becoming enriched in these components. This can be explained by observations on the partially extracted flakes, which suggest that the column of flakes acts much like the stationary phase of a chromatography column, with the SC-CO_2 eluting the triglycerides to a considerable extent before elution of the pigments and other unsaponifiables (Gómez *et al*., 1996).

Chuang and Brunner (2006) reported the concentration of minor constituents in palm oil using SC-CO_2. An enrichment of 550 to 105 000 ppm β-carotene and an enrichment of 300 to 30 000 ppm of sterols were obtained after three steps of extraction (transesterification, followed by two consecutive extractions). The higher rates of extraction of compounds containing polyunsaturated fatty acids than monounsaturated and saturated fatty acids at the beginning of extraction has also been reported in canola oil. (Przybylski *et al*., 1998). These differences in rates of extraction of different compounds during SC-CO_2 extraction may also be attributed to the different solubilities of these compounds in SC-CO_2 (Brunetti, Daghetta, Fedeli, Kikic & Zanderidghi, 1989).

A resultant effect of the different rates of extraction of different compounds during SC-CO_2 extraction of plant oils is the influence on oxidative stability of the oil. Przybylski *et al*., (1998) reported a slight increase in triglyceride content and decrease in free fatty acids from 2.03 to 0.73% between the first and last fractions of canola oil extracted with SC-CO_2. The contents of phospholipids increased as the extraction progressed and the total phospholipid content increased by a factor of 28 in the last fraction, compared to the first (Przybylski *et al*., 1998). The first fractions of the canola oil had lower oxidative stability, and this was attributed to their higher

contents of polyunsaturated fatty acids, higher levels of free fatty acids and absence of phospholipids. The most oxidatively stable canola oil fraction (the last fraction), contained the highest amount of phospholipids and sterols (Przybylski *et al*., 1998).

1.2.5 Lipid oxidation

Lipid oxidation is one of the major causes of food spoilage and is of great concern to the food industry, as it leads to the development of off-odours and decreases the nutritional value of food (Nawar, 1985). It is generally agreed that reaction with molecular oxygen and subjection to elevated temperatures are the main factors influencing the oxidative deterioration of lipids. In order to produce a healthier product for the consumer, the shelf life or oxidative stability of vegetable fats is the top priority for the oil manufacturer (Gunstone, 1996).

Lipid oxidation can be divided into three steps: Initiation, Propagation and Termination (McClements & Decker, 2000). During initiation, highly reactive free radicals are created when oxygen reacts with a substrate (fatty acids) (reaction equation 1).

Initiation $O_2 + RH \longrightarrow R^{\bullet} + HOO^{\bullet}$ …1

These free radicals are highly reactive in their short lifespan in search of another unpaired electron (Gunstone, 1996). The initiation step cannot be stopped by additives. Only the exclusion of radical formers can inhibit free radical production. During propagation, the atmospheric oxygen reacts with the free radicals to form peroxy radicals ($ROO^{\bullet}$) (reaction equation 2). These highly reactive free radicals go on to react with other unsaturated fatty acids where a hydrogen (H)-atom is removed from a fatty acid molecule to form hydroperoxides or primary oxidation products (ROOH) (reaction equation 3), which are odourless and tasteless. This H-abstraction is the slowest and, hence, the limiting step in radical ($R^{\bullet}$) formation (Belitz, Grosch & Schieberle, 2004[a]). This second step in the propagation process (reaction equation 3) will continue until oxygen is depleted or a reaction with a stable antioxidant occurs. Peroxidation of unsaturated fatty acids (reaction equation 3) is

accelerated autocatalytically by radicals generated from the degradation of hydroperoxides by a monomolecular reaction mechanism (reaction equation 4). The degradation of hydroperoxides is prompted by heavy metal ions (Berger, 1994) and is considered to be a starting point for the formation of volatile reaction products. The latter are usually powerfully odorous compounds (consisting of ketones, aldehydes, alcohols and acids created by either peroxide scission alone or simultaneous peroxide and chain scission) perceived as rancidity by the consumer. After a while, the hydroperoxide concentration reaches a level at which it begins to generate free radicals by a bimolecular degradation mechanism (reaction equation 5). However, in food products, reaction equation 5 is of no relevance, since lipid oxidation makes food unpalatable well before reaching the necessary hydroperoxide level for this to occur.

Propagation	$R^{\cdot} + O_2$	$\longrightarrow$	$ROO^{\cdot}$	…2
	$ROO^{\cdot} + RH$	$\longrightarrow$	$ROOH + R^{\cdot}$	…3
	$ROOH$	$\longrightarrow$	$RO^{\cdot} + {}^{\cdot}OH$	…4
	$ROOH$	$\longrightarrow$	$RO^{\cdot} + ROO^{\cdot} + H_2O$	…5

The increase in free radicals leads to them reacting with each other to form stable end products. This reaction step is known as termination (reaction equation 6) and usually plays a role when the oxygen level is low.

Termination	$R^{\cdot} + RO^{\cdot} + ROO^{\cdot}$	$\longrightarrow$	Stable Species	…6

Factors influencing oxidation include oxygen concentration, chemical structure of lipids including unsaturation and chain length, packaging, metal ions, moisture, light, temperature and antioxidants. Oxidation can be inhibited during the initiation step and it is therefore important to ensure correct processing and storage conditions which minimize or eliminate these factors. The addition of an antioxidant will prevent the breakdown of hydroperoxides by inhibiting the propagation process and subsequently prevent rancidity by breaking the oxidation chain. These chain breaking antioxidants include tocopherols, polyphenols, carotenoids and flavonoids

(Murcia, Jiménez, & Martínez-Tomé, 2001). These antioxidants can also occur in avocado oil and will therefore influence the oxidative stability depending on the combination and concentration of these compounds in the oil.

1.2.6 Factors affecting oxidative stability of avocado oil

The oxidative stability of avocado oil can be influenced by various factors. These include the chemical composition of the oil (unsaponifiables, saponifiables, metals), ripeness of the avocado fruit from which oil is extracted and pre-treatment of the avocado fruit prior to oil extraction.

1.2.6.1 Effect of composition of the unsaponifiable matter on oxidative stability of avocado oil

Unsaponifiable matter refers to those substances frequently found dissolved in fats and oils, which cannot be saponified by alkali treatment, but are soluble in ordinary fat and oil solvents (Farines *et al*., 1995). These compounds include higher aliphatic alcohols, sterols, tocopherols, carotenoids, pigments and hydrocarbons (Farines *et al*., 1995). The analysis of the unsaponifiable fraction of vegetable oils is widely recognized as crucial in determining their origin and possible adulteration (Frega, Bocci, Giovannoni & Lercker, 1993). It is also important in predicting the oxidative stability because complementary to the fatty acid profile, it gives an indication of the antioxidant potential inherent to the oil. This is often based on antioxidants (tocopherols, carotenoids) or prooxidants (metal ions) present in the unsaponifiable fraction.

According to Lozano *et al*. (1993) the unsaponifiable matter in avocado oil extracted from young fruit was higher than for mature fruit. In oil extracted from fresh avocado fruit, the unsaponifiables amounted to 1 – 2 % compared to the 3 – 7 % in oil extracted from dried avocado fruit (Farines *et al.*, 1995). This difference is associated with the formation of a new class of compounds, consisting of a long aliphatic chain attached to a furyl nucleus, which accounts for up to 50 % of the unsaponifiable part. The presence of these components can be linked with

pharmacological activity of the unsaponifiable part of the avocado lipids and is formed during heating before extraction (Farines *et al.*, 1995). The unsaponifiable matter of avocado oil is currently used in various pharmaceutical and cosmetic preparations (Farines *et al.*, 1995). The inclusion of the seed during extraction increases the unsaponifiable fraction as the seed contains 55% unsaponifiable material (Werman & Neeman, 1987).

Lozano *et al*. (1993) analyzed avocado oil using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) and measured decreases in unsaponifiable matter in commercial crude avocado oil after industrial treatment. Four major groups of peaks were observed for the unsaponifiable fraction of avocado oil namely, hydrocarbons, tocopherols and sterols (Δ-5 and Δ-7 sterols). HPLC was not selective enough to separate all the fractions of the unsaponifiable matter together with the individual sterol molecules contained in each of these fractions in a single run.

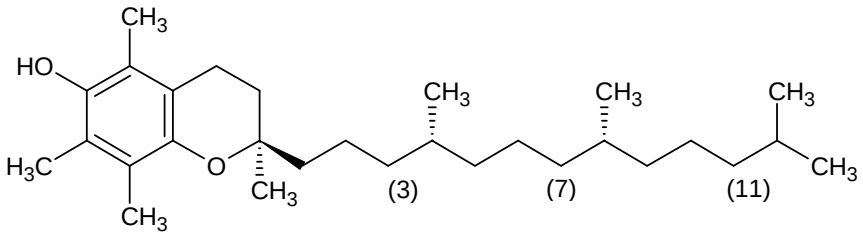
1.2.6.1.1 Tocopherols (Vitamin E)

The methyl derivatives of tocol are denoted tocopherols (Belitz *et al*., 2004[a]). Tocols are 2-methyl-2(4',8',12'-trimethyltridecyl)chroman-6-ols. Tocotrienols are identical except for the occurrence of double bonds at positions 3', 7', and 11' in the side chain (Gregory, 1996) (Figure 1.3). All the isomers of vitamin E are pale yellow, clear, viscous, oily substances, with a boiling point of 200-220 ºC. The eight naturally occurring isomers are α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol (Bramley, Elmadfa, Kafatos, Kelly, Manios, Roxborough, Schuch, Sheehy & Wagner, 2000). The isomers of tocopherols and tocotrienol differ according to the number and position of the methyl groups and thus differ significantly in vitamin E activity (Gregory, 1996).

Vitamin E is a fat-soluble vitamin and is present in nearly all food materials, but found at high concentrations in vegetable oils, nuts, plant tissues and fruits (Tawfik

& Huyghebaert, 1997). The richest sources of vitamin E in the human diet are vegetable oils and the products made from them (Bramley *et al*., 2000).

a)



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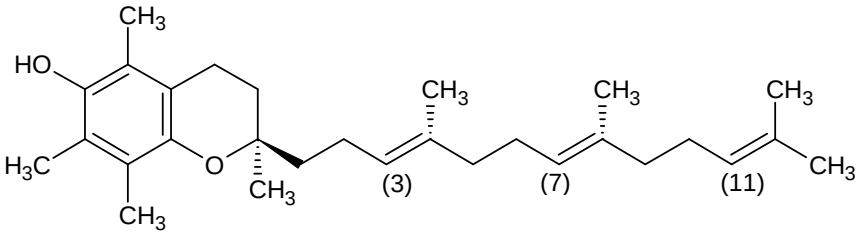


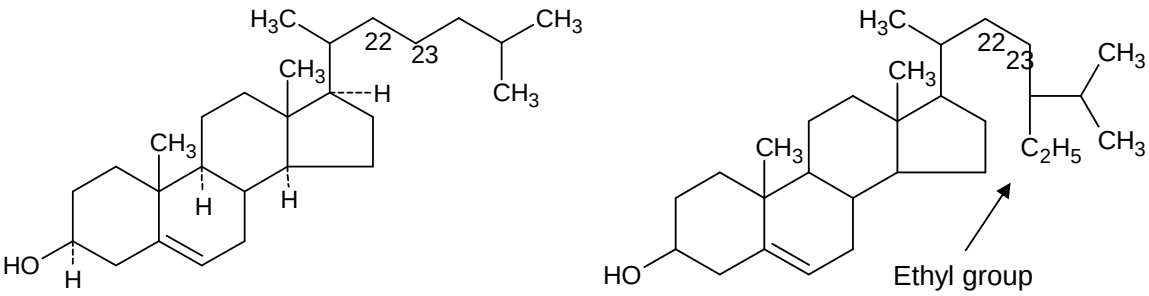
Figure 1.3: Tocopherol (a) and Tocotrienol (b)

The tocopherol content of crude oil is higher than that of refined oils due to losses during deodorization (O'Brien, 1998). Good sources of vitamin E in refined oils include wheat germ (257 mg/100 g), maize (168 mg/100 g) and walnut oil (161 mg/100 g) (Gunstone, 1996). In comparison, crude avocado oil has a tocopherol content of 5.7 – 10.3 mg/100 g oil for mature fruit and 20.1 – 45.6 mg/100 g oil for immature fruit. No significant variation has been detected between the tocopherol content of the varieties *Zutano*, *Bacon*, *Fuerte* and *Lula* (Lozano *et al.*, 1993).

Tocopherols are the best known and most widely used antioxidants (Murcia *et al*., 2001). Tocopherols function as antioxidants by donating the hydrogen of the hydroxyl group to the lipid peroxyl radical. The hydrogen-donating power of tocopherols in fats and oils is in the order δ>β~γ>α (Murcia *et al*., 2001). Tocopherols can also function as inhibitors of lipid oxidation by scavenging singlet oxygen molecules (Kamal-Eldin & Appelqvist, 1996; Fukuzawa, Inokami, Tokumura, Terao & Suzuki, 1998) and free radicals (Schuler, 1990).

1.2.6.1.2 Plant Sterols

Sterols are crystalline, neutral, unsaponifiable alcohols of high melting points with properties resembling those of cholesterol (Sonntag, 1997[a]). The chemical structures of these sterols differ from cholesterol only by an additional methyl-group (campesterol) or ethyl-group (sitosterol) at the C24-position (Figure 1.4), or by an additional double bond at the C22-position (stigmasterol) (Jansen, Lüjohann, Abildayeva, Vanmierlo, Plösch, Plat, Von Bergmann, Groen, Ramaekers, Kuipers & Mulder, 2006). This small structural difference leads to very divergent metabolic fates of plant sterols and cholesterol in mammals including the lowering of serum cholesterol which is attributed to plant sterols (Gylling, Radhakrishnan & Miettinen, 1997).



a.) b.)

Figure 1.4: Typical sterol structure represented by (a) cholesterol and (b) β-sitosterol (Przybylski, 2006)

Plant sterols comprise the bulk of the unsaponifiable matter in many fats and oils and are found in relatively large amounts in nuts and avocados (Jansen *et al*., 2006). Plant sterols, also called phytosterols have been reported to include over 250 different compounds. Although sitosterol is the main sterol in plant materials, stigmasterol, campesterol, brassica- and avenasterols are generally also present (Piironen, Toivo & Lampi, 2000).

The sterol content is less in the crude oil of mature avocado compared to immature fruit (Lozano *et al.*, 1993). This is a direct consequence of the tremendous drop of unsaponifiable content in the oil between the two stages of maturity of the fruits. According to Lozano *et al*. (1993), hexane-extracted oil from freeze dried immature *Fuerte* avocados contain 1.1% sterols, while oil extracted from mature fruit contain 0.9% sterols. The composition of the sterol fraction of avocado oil is given in Table 1.2.

Table 1.2: Composition (%) of the sterol fraction of avocado oil calculated on the basis of the high resolution gas chromatography (HRGC) peak areas (Frega *et al*., 1993).

Sterols	**Percentage (%)**
Cholesterol	0.6
X1*	1.2
Campesterol	2.8
X2*	0.5
Stigmasterol	0.2
B-sitosterol	81.4
X3*	Trace
X4*	Trace
Δ^5-avenasterol	9.0
Δ^7-stigmasterol	Trace
X5*	Trace
Δ^7-avenasterol	Trace
Other	4.3

* Fractions not identified

Due to the presence of oxidatively sensitive hydrophilic hydroxyl groups and double bonds in their chemical structure, phytosterols are susceptible to oxidation in oils and food products (Dutta, 1997). Several studies have confirmed the stabilizing effect of some sterols in oil subjected to prolonged heating (Sims, Fioriti & Kanuk,

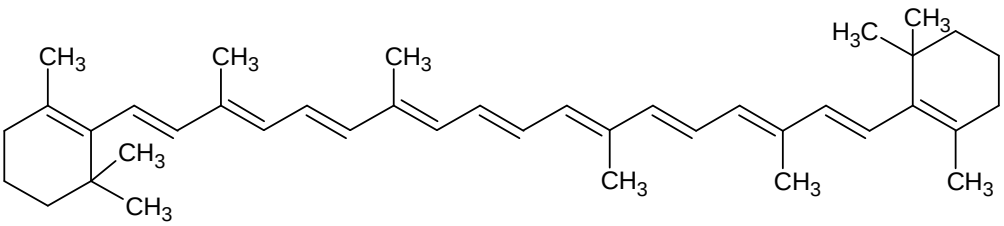
1972; Boskou & Morton, 1976; White & Armstrong, 1986). Sterols such as fucosterol, Δ-5 avenasterol, vernosterol, fucosterol and citrostadienol have been shown to have antioxidant activity in oils at 180ºC (Rajalakshmi & Narasimhan, 1995; White & Armstrong, 1986) whereas stigmasterol and cholesterol did not exhibit any antioxidant activity (Gordon & Magos, 1983). It has also been suggested that sterols function by forming a monolayer at the surface of oils to inhibit oxidation by acting as hydrogen donors. However, Cercaci, Tassalcqua, Poerio, Rodriguez-Estrada & Lercker (2007) found a minor, non-significant effect of total sterols obtained from extra virgin olive oil on the oxidative stability of vegetable model systems when determined by the oxidative stability index (OSI). They focused on considering the synergistic or antagonistic effects of the various sterols, thus hiding their single antioxidant properties, which might provide a more realistic picture of the overall antioxidant capacities of sterols.

1.2.6.1.3 Carotenoids

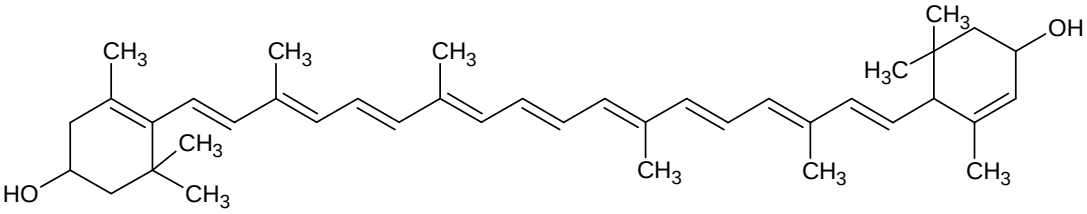
Carotenoids are structurally unique molecules consisting of a system of conjugated double bonds and are responsible for the yellow, orange or red colour in plants. They have a 40-C skeleton and consist of eight isoprene units, arranged in a head-to-tail manner to create a symmetrical molecule (Stahl & Sies, 1996). Carotenoids are divided into two main classes: carotenes and xanthophylls (Belitz *et al*., 2004[a]). Xanthophylls contain oxygen in the form of hydroxyl, epoxy or oxo groups, while carotenes are pure polyene hydrocarbons (Belitz *et al*., 2004[a]) (Figure 1.5). Carotenoids are present in plants as a complex mixture and often occur as esters of fatty acids (Belitz *et al*., 2004[a]).

The carotenoid content of the avocado is higher in the yellow part of the mesocarp than in the green part under the skin. It was this obvious yellow-green colour, normally attributed to lutein, which prompted Lu, Arteaga, Zhang, Huerta, Go & Heber (2005), to quantify the carotenoids present in the mesocarp of the avocado and specifically investigate the lutein content. Among five carotenoids measured

namely: α- and β-carotene, lutein, zeaxanthin and β-cryptoxanthin, lutein accounted for 70% of total caotenoids.



a.)



b.)

Figure 1.5: Examples representing the two main classes of carotenoids (a) ß-carotene (representing the carotenes) and (b) Lutein (representing the xanthophylls) (Belitz *et al*., 2004[a])

Although seasonal variation was observed, contents of lutein varied from 232 to 335 µg/100g fruit. The USDA-NCC Carotenoid Database for US Foods (2002) indicates a β-carotene content of 34 µg/100g and a lutein content of 320 µg/100g for avocado oil. According to Gross, Gabai and Lifshitz (1972;1973), lutein and chrysanthemaxanthin each comprise about 21-25% respectively, of the total carotenoids of avocado mesocarp, while the other types of carotenoids each comprise about 1-10%. Ashton *et al*. (2006) reported a decrease in the total carotenoid content of peel (200-100 mg/kg) and mesocarp (green flesh: 23-10 mg/kg and yellow flesh: 15-1 mg/kg) during ripening, in oil extracted from freeze dried *Hass* avocado.

Carotenoids are able to deactivate radical-mediated reactions and thus inhibit lipid peroxidation. They may also protect against the formation of singlet oxygen by

preventing exposure to light through their function as a natural light filter, which is effective for wavelengths from 400 nm to 500 nm (Zambiazi & Przybylski, 1998). Among the various defence strategies, carotenoids are most likely involved in the scavenging of two of the reactive oxygen species, singlet moleculer oxygen and peroxyl radicals. Carotenoids are sensitive to oxygen and light and are stable in food even at high temperatures, when these two factors are excluded (Belitz *et al*., 2004[a]). Suzuki and Shioi (2003), however observed a structural change of carotenoids due to heat as well as a decrease in lutein after the heat treatment involved in the processing of Japanese teas (Suzuki & Shioi, 2003). It has been established by Chen and Chen (1993) that epoxy-containing carotenoids are more susceptible to heat than other carotenoids. Warner and Frankel (1987) reported that the presence of 5 to 20 ppm of β-carotene had a significant effect in protecting soybean oil against light deterioration. In a comparative study on carotenoids and tocopherol the order of antioxidant potency was described in the following order: α-tocopherol > α-carotene > lutein > zeaxanthin = β-carotene (Farombi & Britton, 1999). All carotenoids however, showed antioxidant potential by significantly reducing the rate of peroxyl formation.

1.2.6.1.4 Chlorophyll

Chlorophylls are magnesium complexes derived from porphin which is a fully unsaturated macrocyclic structure that contains four pyrrole rings linked by single bridging carbons (Von Elbe & Schwartz, 1996) (Figure 1.6). According to Von Elbe and Schwartz (1996), chlorophylls are the major light-harvesting pigments in green plants and other photosynthetic organisms. Several chlorophylls and their derivatives are found in nature including chlorophyllides, pheophorbides, pheophytins and pyropheophytins (Gunstone, 2004). The level of pheophytin, is an important criterion in determining the quality of crude oils, especially canola oil (Daun, 1982).

Refined avocado oil has a chlorophyll content of 0.3 mg/kg (Smith & Winter, 1970), while the crude oil has chlorophyll values of 40-60 mg/kg (Eyres *et al*., 2001).

Chlorophyll seems to decrease with fruit ripening (Ashton *et al*., 2006). A decrease of 214 to 116 mg/kg oil in total chlorophyll content of oil extracted from freeze dried *Hass* avocado peel during ripening (14 days at 20℃) has been observed (Ashton *et al*., 2006).

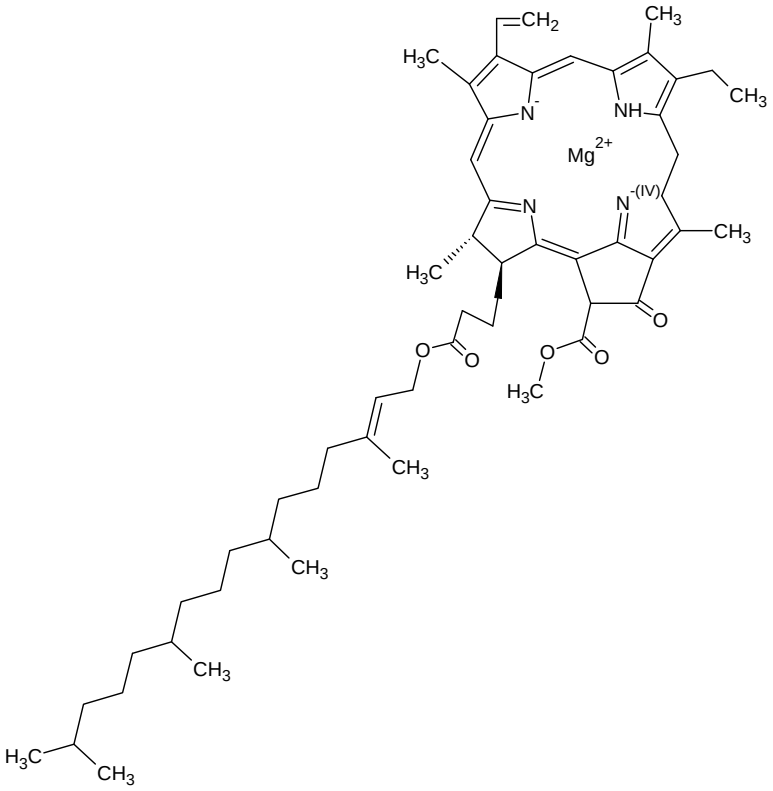


Figure 1.6: Chlorophyll, with the active porphyrin site surrounding the Mg^{2+} complex (Belitz *et al*., 2004[b])

Chlorophyll derivates are formed during processing due to heat, acid and enzymatic actions (Figure 1.7). The formation of pheophytin *a* due to heat has been well established (Suzuki & Shioi, 2003). Chen and Chen (1993), showed that when sweet potato leaves were heated briefly in a microwave, the concentrations of epimers of chlorophylls *a* and *b* and pheophytin *a* increased. Epimer formation of chlorophylls *a* and *b* in tea leaves is considered to be due to heating during processing of tea (Suzuki & Shioi, 2003). Chlorophyll *a* is more susceptible to heat

and the conversion rate of chlorophyll *a* to pheophytin *a* is higher than that of chlorophyll *b* to pheophytin *b*.

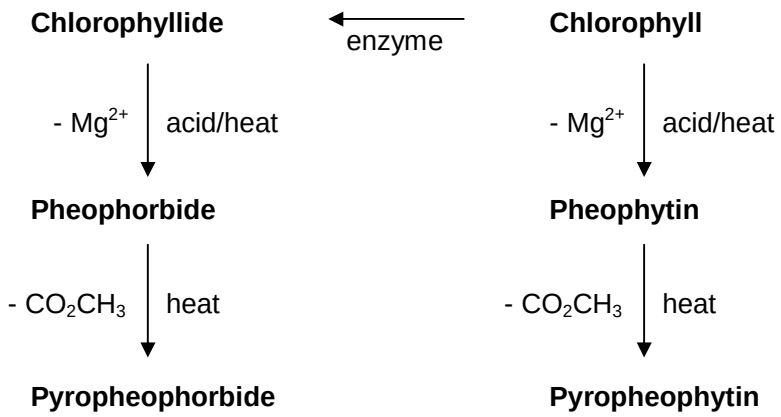


Figure 1.7: Formation of chlorophyll derivatives due to heat, acid and enzymes (Von Elbe & Schwartz, 1996)

Chlorophyll is a strong prooxidant. The oxidation reaction, in which chlorophyll acts as oxidising agent, is catalyzed by light (Smouse, 1995). The oxidative stability of vegetable oils is greatly affected by the presence of chlorophylls and their derivatives which have the ability to transfer energy from light to other molecules (Foote, 1979). The most detrimental product formed during this transfer of energy is singlet oxygen, which initiates the oxidation of oils. Oxidation then proceeds by an ene reaction forming a *trans* configuration of an unsaturated hydroperoxide (Frankel, Neff, Selke & Weisleder, 1982). These reactions are not affected by antioxidants, but can be inhibited by singlet oxygen quenchers such as carotene.

Chlorophyll however, seems to have antioxidant potential when the autoxidation reaction occurs in the dark (Endo, Usuki & Kaneda, 1984; Endo *et al*., 1985[a]; Zambiazi & Przybylski, 1998; Psomiadou & Tsimidou, 2002). Chlorophyll, as well as its derivatives, such as pheophytin, protoporphyrin methyl ester and magnesium chelated porphyrin methyl ester have been proven to retard the formation of peroxides and carbonyl compounds during autoxidation of methyl linoleate in the

dark at 30ºC (Endo *et al*., 1985[b]). It was concluded that the antioxidant effect of chlorophyll may not be ascribed to the decomposition of hydroperoxides, but rather to the chain breaking reaction by donating electrons to reduce free radicals. The essential structure for antioxidant activity of chlorophyll derivatives was found to be the porphyrin compounds, possibly strengthened by magnesium, but only in the chelated form. Antioxidant activities of chlorophyll and pheophytin were also demonstrated in oven tests with magnesium linoleate as substrate (Endo *et al*., 1985[a]).

1.2.6.2 Effect of composition of the saponifiable matter on oxidative stability of avocado oil

Saponifiable matter refers to lipid substances that can be saponified by caustic treatment. This fraction usually consists of the triglycerides and phospholipids.

1.2.6.2.1 Fatty acids

A fatty acid is a carboxylic acid often with a long unbranched aliphatic tail (chain), which is either saturated (no double bonds) or unsaturated (one or more double bonds) (Gunstone, 1996). Saturated fatty acids form straight chains, while unsaturated ones can take up different forms. Avocado oil is classified as a monounsaturated oil together with other oils such as olive, apricot kernel and macadamia (Gunstone, 1996). The predominant fatty acids in avocado oil are oleic acid (65-75%) (Figure 1.8), linoleic acid (10-18%) and palmitic acid (12-18%) (Werman & Neeman, 1987).

The fact that the oil is more mono-unsaturated will promote its oxidative stability (Werman & Neeman, 1986) in contrast to polyunsaturated oils such as sunflower and grape seed oil. The ease and rapidity with which an oil oxidizes depend primarily on the number of double bonds of the fatty acids and their arrangement (Sonntag, 1979[b]).

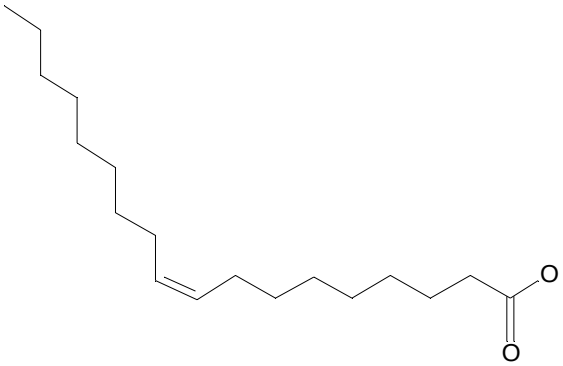


Figure 1.8: Oleic acid, a monounsaturated fatty acid with one double bond

Polyunsaturated fatty acids are oxidized more rapidly than monounsaturated or saturated fatty acids resulting in polyunsaturated fatty acids being the focal point in autoxidation of fats and oils (Sonntag, 1979[b]). The main polyunsaturated component of principal edible oils is linoleic acid and therefore the mechanism of autoxidation of this acid is of major importance in oxidative rancidity (Sonntag, 1979[b]). The rate of autoxidation of methyl interrupted polyunsaturated systems is much higher than that of monounsaturated systems because a methylene group is activated by surrounding double bonds. According to Gunstone (1996), the rates of oxidation of methyl oleate, methyl linoleate and methyl linolenate are 1:12:25. Being a monounsaturated oil, containing high amounts of oleic acid, avocado oil will oxidise slower than polyunsaturated oils, containing more linoleic or linolenic acid.

1.2.6.2.2 Phospholipids

Phospho- and glycolipids, together with proteins, are the building blocks of biological membranes (Garret & Grisham, 1995). They are surface-active compounds and contain hydrophobic moieties and hydrophilic portions, forming bilayer structures in all biological membranes. Crude oils generally contain phospholipids, which are removed during refining at the degumming stage (Gunstone, 2004). The major components are phosphatidylcholines (Figure 1.9), phosphatidylethanolamines and phosphatidylinositides accompanied by smaller proportions of other phospholipids (Gunstone, 2004).

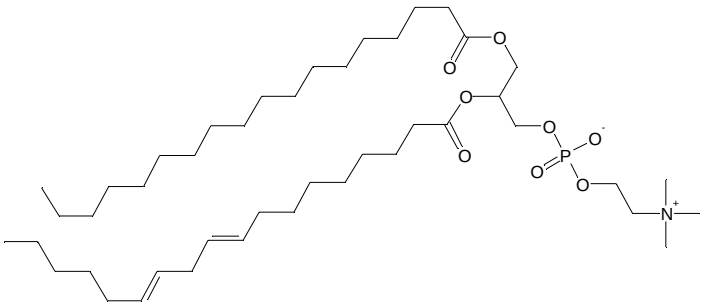


Figure 1.9: Phosphatidylcholine (lecithin), extracted from soybeans

According to Du Plessis (1980), *Fuerte* contained much higher phospholipid concentrations when compared to other avocado varieties. The phospholipid content was also proven to be higher in solvent extracted avocado oil (4.0-7.2%) than in centrifugal extracted avocado oil (0.4-2.5%) (Du Plessis, 1980).

The synergistic antioxidative effects of phospholipids are well documented (Chu, 1991; Hara, Okada, Hibino, & Totani, 1992). Various antioxidative mechanisms have been proposed for phospholipids, for example metal-chelating properties (Zambiazi & Przybylski, 1998), oxygen barrier effect, assisting in the dispersion of other antioxidants in emulsion systems as well as limiting the propagation of free radicals in the medium (Hamilton, Kalu, McNeill, Padley & Pierce, 1998). Furthermore, when heating oil at high temperatures, aldehydes can form complexes with phospholipids through carbonyl and amino group interactions and generate malanophosphatides, which themselves inactivate hydroperoxides (King, Boyd & Sheldon, 1992[a]). A synergistic effect of lecithins with phenolic antioxidants (King, Boyd & Sheldon, 1992[b]) as well as tocopherols (Judde, Villeneuve, Rossignol-Castera & Le Guillou, 2003) has also been observed. The addition of standard crude lecithin (containing approximately 60% phospholipids) was used by Judde *et. al.* (2003) to illustrate the synergistic effect of phospholipids during lipid oxidation. When tested with the Rancimat (accelerated oxidation test) rapeseed oil alone had an induction time of 8.4 hours, while this time increased to 37.6 hours, with the addition of 5% crude lecithin (Chu & Hsu, 1999).

1.2.6.3 Effect of avocado fruit ripeness on oxidative stability of avocado oil

Unripe avocado fruit is used for the pressing of avocado oil (Carr, 1997), while ripe fruit is used for the centrifugal (Benedito *et al*., 2004) and solvent extraction of the oil (Owusu-Ansah, 1997). There is a distinct difference between maturity of fruit and ripeness. The avocado is a climacteric fruit, and only starts to ripen after harvesting (Awad & Lewis, 1980; Sippel, 2001; Ozdemir & Topuz, 2004). Ripening is often completed 5 – 7 days following harvest (Seymore & Tucker, 1993) although Ozdemir and Topuz (2004) allowed 8 days at 18 – 22°C for ripening of avocado fruit when they investigated the changes in fatty acid composition. Ripening is accompanied by physical and chemical changes in the fruit and it is the chemical changes that can influence oxidative stability of oil extracted from the fruit.

Ripening might have a negative effect on the oxidative stability as the micro-components known for their antioxidant potential decrease during ripening. Ashton *et al*. (2006) reported a decrease in the total carotenoid content of peel and mesocarp during ripening, in oil extracted from freeze dried *Hass* avocado. These authors also observed a decrease in total chlorophyll content of oil extracted from freeze dried *Hass* avocado peel during ripening (14 days at 20℃). T he decrease of chlorophyll, on the other hand might increase the oxidative stability.

1.2.6.4 Effect of avocado fruit pre-treatment on oxidative stability of avocado oil

The drying method or pre-treatment and storage of avocado fruit prior to extraction plays a large role in the quality and characteristics of the extracted oil. Oven drying is a well-known and relatively cost-effective drying technique used in the oil industry prior to mechanical pressing. This can be both detrimental and advantageous where oxidative stability of oil is concerned, as heat is known to destroy tocopherols, but elevated temperatures also inactivate enzymes that lead to hydrolysis of fatty acids from triglycerides (Gunstone, 1996). Freeze drying on the other hand, is a milder drying technique where the product is not exposed to elevated temperatures and has been proven to preserve phenolic (Ferreira, Nogueira, Souza & Batista, 2004) and carotenoid (Çinar 2004) compounds better than oven drying. Although

freeze drying would seem like a superior drying technique it is important to note that the drying temperatures are too low to destroy enzymes which could lead to hydrolysis of fatty acids from glycerol (Belitz *et al*., 2004[a]). According to Çinar (2004), phytochemicals like phenols are best preserved in freeze dried samples and storage in dark, cool places is advised. Refrigeration is recommended (Ferreira *et al*., 2004), but this is often difficult to execute in industry.

Heat treatment of avocado fruit prior to oil extraction seems to lead to the formation of a compound consisting of a furyl nucleus fixed to a linear hydrocarbon-based saturated or unsaturated chain comprising one or more ethylenic or acetylenic unsaturations (Rancurel, 1993). This compound has, however, not been proven to have antioxidant activity.

Extraction method of oil is closely linked to the pre-treatment (fruit ripeness and drying method in this study) of the fruit prior to extraction and the quality of the oil is often already established before extraction commences.

1.2.7 Hypotheses

1. Oil yield obtained from avocado fruit will be influenced by ripeness of the fruit, drying method prior to oil extraction and the extraction method used. Enzymatic degradation of cellular structure during ripening will expose oil cells in ripe fruit and make them more accessible, leading to higher oil yields in ripe fruit compared to unripe. More oil will be extractable from freeze-dried fruit than from oven-dried fruit because plant material will be more porous after freeze-drying than after oven-drying, where denaturing (Belitz *et al*., 2004[c]) and cross-linking of proteins (Duodu, Taylor, Belton, & Hamaker, 2003) and starch, will form barriers around oil cells in the fruit, which will decrease the extractability. Hexane extraction will produce a higher oil yield than SC-CO_2 extraction. Hexane is less selective as an extraction solvent (Gómez & de la Ossa, 2002) compared to SC-CO_2 and will extract a higher content of unsaponifiables leading to higher oil yield for hexane extracts.
2. The micro-component (tocopherols, sterols, carotenoids, chlorophyll) content of the avocado oil extracted from ripe fruit will be lower than the micro-component content of oil extracted from unripe fruit (Ashton *et al.,* 2006) because enzymes such as lipoxygenases which are known to reduce the tocopherol, carotenoid and chlorophyll levels, increase in ripe avocado fruit compared to unripe. The tocopherol, carotenoid and sterol content of the oil extracted from oven-dried fruit will be lower than that of the oil extracted from freeze-dried fruit because these components might be oxidised by the heat treatment (80℃) during oven-drying. Progressive e xtraction will yield an oil enriched in chlorophyll and carotenoids, while the sterol and tocopherol content will remain unchanged throughout progressive extraction. This difference in separation is based on a lower initial availability of the pigments in the plant material. Tocopherols and sterols form part of the cell membrane (Kumar, Raclaru, Schüßeler, Gruber, Sadre, Lühs, Zarhloul, Friedt, Enders, Frentzen & Weier, 2005; Taiz & Zeiger, 2006) where they are readily available for extraction, while chlorophyll and carotenoids are located in

chloroplasts and chromoplasts respectively where thick cell walls will make them less available during the first phases of progressive extraction.

3. The oxidative stability of the oil extracted from unripe fruit will be higher than that of oil extracted from ripe fruit because the antioxidants which protect the oil (carotenoids, tocopherol) will be present at higher levels in unripe fruit and lower levels in ripe fruit due to oxidation by increased levels of lipoxygenase enzymes during ripening (Lajolo & Anfer-Marquez, 1982; Lopez-Ayerra, Murcia & Garcia-Carmona, 1998; Ashton *et al.*, 2006). The oxidative stability of oil extracted from freeze-dried fruit will be higher than that of oil extracted from oven-dried fruit because freeze-drying is a milder drying technique which eliminates the exposure of the fruit to high temperatures which is known to increase the oxidative and hydrolytic deterioration rate of oils (Berger, 1994). Progressive extraction will yield an oil with an increased oxidative stability due to the absence of free fatty acids and an increase in micro-components with antioxidant activity, including carotenoids, in the last fractions (Przybylski *et al*., 1998).

1.2.8 Objectives

1. To determine the oil yield obtained from SC-CO_2 or hexane extraction of avocado as influenced by fruit ripeness and method of fruit drying (freeze-drying or oven-drying).
2. To determine the micro-component (tocopherols, sterols, carotenoids, chlorophyll) content of avocado oil extracted with SC-CO_2 and how these are influenced by fruit ripeness, method of fruit drying and progressive extraction.
3. To determine the oxidative stability of avocado oil extracted with SC-CO_2 as influenced by fruit ripeness, method of fruit drying, progressive extraction and micro-component content.

2. RESEARCH

2.1 EFFECT OF FRUIT RIPENESS AND METHOD OF FRUIT DRYING ON THE EXTRACTABILITY OF AVOCADO OIL WITH HEXANE AND SUPERCRITICAL CARBON DIOXIDE*

ABSTRACT

Oil yield from avocado fruit may be influenced by fruit pre-treatment and extraction method. Unripe and ripe avocado fruit pieces were deep frozen at -20^{o}C and either freeze-dried or oven-dried (80^{o}C). Oil yield from these samples was determined after extraction with hexane and supercritical carbon dioxide (SC-CO_2). The fruit samples were examined using scanning electron microscopy before and after oil extraction. Average oil yield from ripe fruit (freeze-dried and oven-dried combined) was 72 g kg^{-1} higher than from unripe fruit for SC-CO_2 extracts and 61 g kg^{-1} higher for hexane extracts. This may be due to enzymatic degradation of parenchyma cell walls during ripening, thus making the oil more available for extraction. Freeze-dried samples had a mean oil yield 55 g kg^{-1} greater than oven-dried samples for SC-CO_2 extracts and 31 g kg^{-1} higher for hexane extracts. However, oil yields from ripe fruit (freeze-dried and oven-dried) subjected to hexane extraction were not significantly different. All hexane extracts combined had a mean oil yield 93 g kg^{-1} higher than SC-CO_2 extracts. The SC-CO_2 may be more selective and may create paths of least resistance through the plant material. Hexane on the other hand, is less selective and permeates the whole plant material leading to more complete extraction and higher oil yields under the experimental conditions.

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2.1.1 Introduction

Avocado oil has valuable nutritional properties, including its high level of monounsaturated lipids known for lowering blood cholesterol levels. The oil is also used in cosmetic preparations (Knight, 2000) and its unsaponifiable matter is exploited in various pharmaceutical applications for therapeutic, dermatological and medical uses (Henrotin, Sanchez, Deberg, Piccardi, Msika, & Reginster, 2003; Neeman, Lifshitz & Kashman, 1970; Rancurel, 1985).

In the avocado fruit, oil is situated in the mesocarp (Somogyi *et al*., 1996) which consists primarily of large parenchyma cells, idioblast cells and a network of vascular strands (Scott *et al*., 1963). Parenchyma cells mainly contain numerous droplets of lipid substances, mostly triacylglycerol (Platt & Thomson, 1992). Idioblast cells have thicker walls and the oil in the cells occur in oil sacs as mostly singular, large drops and has a different composition from the oil in parenchyma cells (Platt-Aloia *et al*., 1983; Werman & Neeman, 1987).

Being a climacteric fruit, the avocado only starts to ripen after harvesting (Awad & Lewis, 1980; Ozdemir & Topuz, 2004). During ripening, the primary walls of the parenchyma cells are degraded due to the activities of cell wall degrading enzymes, namely cellulase and polygalacturonase (Awad & Lewis, 1980; Reymond & Phaff, 1965; Zauberman & Shiffmann-Nadel, 1972). This structural degradation might cause the oil to be liberated from the cellular bodies and become more available for extraction. The suberised wall of the idioblast oil cells is however, immune to the activity of these enzymes and remains intact during ripening (Platt & Thomson, 1992).

Oil content is closely related to horticultural maturity and is often used as an indicator of fruit maturity and harvesting time (Lee, Yound Schiffman & Coggins, 1983; Chen, McCarthy, Kauten, Sarig, & Han, 1993). Oil does not seem to increase after harvesting (Lewis *et al*., 1978; Poiana *et al*., 1999). Ozdemir and Topuz

(2004), reported no significant changes in oil content of a few avocado varieties, including, *Fuerte* after ripening.

Water interferes with the effectiveness of oil extraction and therefore drying of the material to be extracted is a necessary step prior to extraction (Lewis *et al*., 1978; Sun & Temeli, 2006). Freeze-drying is a preferred drying method due to low temperatures and minimal exposure of the material to oxygen. This is, however, costly and cheaper methods of drying such as oven-drying are often implemented to alleviate costs (Alomar, Fuchslocher & De Pablo, 2003). It appears that the method of drying used influences the yield of oil obtainable from the material. Moreno, Dorantes, Galíndez, & Guzmán (2003) reported a decrease in oil yield when temperatures higher than 100℃ were applied to avoc ado pulp. This seems to have been caused by a transformation in cellular structure, probably a hardening of the cell wall due to denaturation of proteins, which in turn, prevented the release of the oil.

Conventional solvents such as hexane (Ortiz *et al*., 2004) and petroleum ether (Lewis *et al*., 1978; Ozdemir & Topuz, 2004) have been used to extract avocado oil. Hexane extraction of an unknown cultivar yielded approximately 59% oil from avocado pulp, leaving the idioblastic oil cells irregularly shaped and rough surfaced (Ortiz *et al*., 2004). An avocado oil yield of 74-75% from the *Fuerte* variety has been obtained using petroleum ether for an extraction time of four hours (Lewis *et al*., 1978). Lewis *et al*., (1978) extracted avocado oil using soxhlet extraction (hexane), homogenization with petroleum ether, homogenization with chloroform/methanol as well as by ball milling with 1-chloronaphthalene and found no difference in oil yield between ripe and unripe fruit, but lower yields for the samples extracted with petroleum ether.

The varying solubility properties of SC-CO_2 make it an ideal solvent for the extraction of plant material (Garcia *et al*., 1996). SC-CO_2 is also biologically safer due to the fact that organic solvents are not recovered completely and reused but losses into the atmosphere occur and cause pollution. To the best of our

knowledge, the only report on SC-CO_2 extraction of avocado oil is the one by Botha and McCrindle (2003) where the effect of gas pressure on oil yield and fatty acid composition was studied. The apparent yield from SC-CO_2 extraction of seed oil is slightly lower than extraction with hexane (Friederich & List, 1982; Gómez & de la Ossa, 2002; Bravi *et al*., 2007). Zaidul, Norulaini, Omar, Smith (2007) reported a yield of 48.9% for palm oil extracted with SC-CO_2, whilst hexane extraction yielded 50.1%. The difference in extractability has been attributed to the lower selectivity of hexane for pigments and phospholipids (Gómez & de la Ossa, 2002). The average gossypol content of hexane-extracted cottonseed oil was 0.242% while that of SC-CO_2-extracted cottonseed oil was 0.015% (Bhattacharjee *et al*., 2007).

The aim of this study was to determine the effect of fruit ripeness and method of fruit drying on extractability of avocado oil using hexane or SC-CO_2, with particular regard to oil yield and fruit microstructure.

2.1.2 Materials and Methods

2.1.2.1 Preparation of avocado fruit for oil extraction

Horticulturally mature avocado fruit (variety *Fuerte*) was obtained from farms in the Soutpansberg area, Limpopo province, South Africa. The fruit was divided into two batches. One batch was allowed to ripen and soften by storing at 23℃ for 7 days. Softness was confirmed on an AT-XTPlus (Stable Micro Systems, London, UK) texture analyzer using a blade to penetrate unpeeled avocado wedges from mesocarp adjacent to the stone towards the pericarp. The force was measured as grams needed to penetrate an avocado at a distance of 38 mm using a blade set with a 5 kg load cell. The test speed was 5 $mm.s^{-1}$, the post test speed was 10 $mm.s^{-1}$ and the data acquisition rate was 200 pps.

The unripe batch was destoned and mechanically cut into pieces of approximately 10 x 10 x 10 mm using a CELME electric slicer (Tracazzano, Italy). The ripened fruit was also cut into pieces of approximately 10 x 10 x 10 mm, using a knife, exercising caution to prevent the formation of a pulp. Due to restricted oven capacity, fruit

pieces had to be preserved by deep-freezing at -20°C and dried in batches. Batches of the unripe and ripe fruit pieces were freeze-dried (Virtis Genesis, SP Industries, Warminster, PA, USA) or oven-dried (Forced draft oven, model 361, Labotec, Johannesburg, South Africa) at 80℃ for 24 h to a moisture content of 2-3%. The moisture content was determined using a Precisa infrared moisture balance (model HA 300, Instrulab Cc, Johannesburg, South Africa). The dried samples were stored at -20℃ until required for oil extraction. Prior to oil extraction, the dried fruit pieces were ground to a particle size < 2 mm using a Kenwood food processor Model PFP 32 (Shanghai, China).

2.1.2.2 Supercritical Carbon Dioxide (SC-CO_2) extraction of avocado oil

Liquid carbon dioxide, purity 99.9995% (Air Products, SA) was used without any further purification as the extraction fluid. An in-house built apparatus was constructed for extraction (Botha & McCrindle, 2003), consisting of a high pressure pump (ISCO 100 DX), a 10 ml extraction cell and a 10 ml preheating column (Keystone Scientific, Bellefonte, PA USA) placed in a Carlo Erba Fratovap (Model 2700, Milan, Italy) gas chromatograph oven.

The method used as described here was optimised by Botha and McCrindle (2003). Extractions were performed at 37℃/350 atm. The flu id flow rate was kept constant at 1.7 ml/min measured at the pump head and controlled by a heated needle valve. All extractions were performed on 7 – 8 g of ground, dried avocado pieces and oil was collected in a glass collection vessel without any solvent to assist collection. Extractions were terminated after an oil yield of less than 1 % was obtained in 1 h. The extractions were performed in triplicate.

2.1.2.3 Hexane extraction of avocado oil

Hexane extractions were performed on a Tecator Soxtec Foss 1042 apparatus, (Hoganas, Sweden). Approximately 5 g of dry and ground fruit pieces was weighed into cellulose thimbles and extracted with 120 ml hexane (Merck AR) for 8 h at about 70ºC in triplicate. After completion of the extraction, the cups containing the oil and

residual solvent were dried to constant mass in an oven at 100℃ to remove any solvent left in the oil.

2.1.2.4 Light microscopy (LM) and scanning electron microscopy (SEM)

LM was conducted on fresh, unripe avocado fruit. Samples were dissected (15 mm from the pericarp of the fresh, unripe fruit) and immediately fixed in 2.5 % glutaraldehyde in a 0.075 M phospate buffer for 24 h at 4℃ (pH = 7.3 ± 0.05). After three rinses in the same buffer, samples were post-fixed with 1% aqueous OsO_4 for 2 h. Samples were then rinsed three times with distilled water, and dehydrated in an ethanol dilution series (30, 50, 70, 90 and 3 x 100 %) for 15 min respectively. Samples were then imbedded in Quetol 651 (Van der Merwe & Coetzee, 1992) and cut using a Reichert Ultracut E ultra microtome. Monitor sections for LM of 1 µm thick were cut and stained with Toluidine Blue O (0.2% Toluidine Blue O in 0.5% Na_2CO_3) and viewed with a Nikon Optiphod (Nikon Instech Co., Kanagawa, Japan). Additional pieces without OsO_4 post-fixation were imbedded in LR White medium, sectioned at 1 µm, stained with toluidine blue as above and viewed.

SEM was conducted on unripe and ripe avocado fruit (fresh and after deep freezing at -20℃), unripe and ripe avocado fruit (after fre eze-drying and oven-drying at 80^{o}C) and on unripe and ripe fruit samples (freeze-dried and oven-dried) after hexane and SC-CO_2 extraction. For fresh and deep-frozen fruit, samples were dissected and fixed in glutaraldehyde in phosphate buffer, post-fixed in osmium tetroxide (OsO_4), rinsed with distilled water and dehydrated in an ethanol dilution series as described above for LM. Samples were then subjected to critical point drying with CO_2 (Biorad E3000, Polaron, West Sussex, UK), before freeze fracturing and mounting on double-sided carbon tape on stubs and coated (15-20 nm) with gold using a Polaron E5200 (Watford, England) coater. Due to the dry nature of the freeze-dried and oven-dried fruit samples as well as the samples after oil extraction, these pieces were mounted without any pre-treatment. The inside of the tissue pieces were viewed with a JSM-840 scanning electron microscope (JEOL, Tokyo, Japan).

2.1.2.5 Statistical analysis

Oil extractions and oil yield determinations were done in triplicate. Statistical analysis was done on Statistica 6.0. The Mann-Whitney non parametric alternative (Freund & Walpole, 1980) was performed on the oil yield results. Standard deviations and p values were determined, with difference considered significant at $p < 0.05$.

2.1.3 Results and Discussion

2.1.3.1 Ultrastructure of unripe and ripe avocado fruit

2.1.3.1.1 Light microscopy

Light microscopy (Fig 2.1.1a) clearly showed the parenchyma cells as well as the idioblast cells filled with one large oil sac. The OsO_4 fixed light microscopy image (Fig 2.1.1b) showed parenchyma cells filled with numerous oil droplets, however, it was not clear whether the idioblast cells contained any lipids, as there was no black staining visible within them. It is, however, possible that the contents of these cells may have been lost during the preparation and cutting of the material for light microscopy.

These idioblast cells also have thicker cell membranes (Rodriguez-Saona *et al*., 1998[a]) to which the single oil-containing sac is attached, which might also cause the entire content of the cell to be removed during slide preparation when the mesocarp is cut, removing the cell contents with the cell membrane.

These images of the mesocarp correspond with those published by Platt & Thompson (1992), where the idioblast cells also did not discolour when stained with OsO_4. The content of the idioblast which mainly consists of terpenoids, differs from that of the parenchyma cells which most likely adds to the resistance against fixing with OsO_4 (Werman & Neeman, 1987; Platt-Aloia *et al*., 1983; Prusky *et al*., 1991; Rodriguez-Saona, Millar & Trumble, 1997; Rodriguez-Saona *et al.*, 1998[a]; Rodriguez-Saona *et al*., 1998[b]).

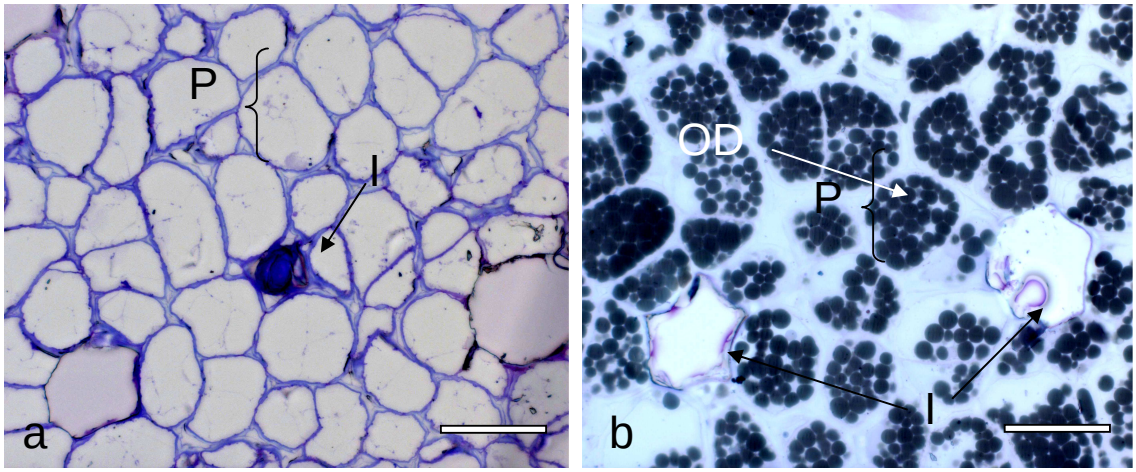


Figure 2.1.1: Avocado mesocarp stained with toluidene blue alone (a) and Avocado mesocarp stained with toluidene blue and fixed with osmium tetroxide. I: Idioblast cells; OD: Oil droplets; P: Parenchyma cell. Bar represents 10 µm.

2.1.3.1.2 Scanning Electron Microscopy

The SEM images of the fresh unripe and ripe fruit are shown in Figures 2.1.2a and 2.1.2b. Parenchyma cells were visible in both the unripe and ripe fruit.

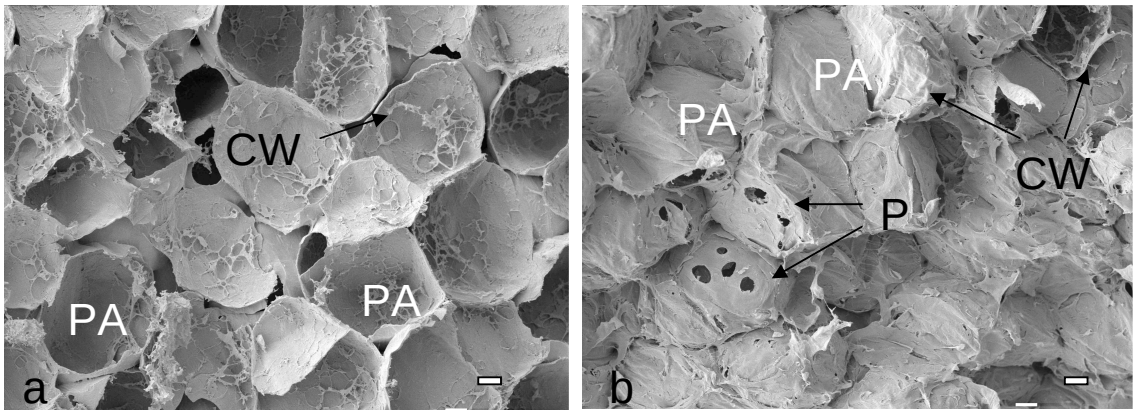


Figure 2.1.2: Scanning electron micrographs of fresh unripe (a) and ripe (b) avocado mesocarp showing parenchyma cells (PA), cell walls (CW) and pits (P). Bar represents 10 µm.

Some of the parenchyma cells were intact while others seemed to be open, especially in the unripe sample, possibly as a result of the sample preparation process. Cell walls were visible on both the unripe and ripe fruit micrographs. Small holes or pits were visible in the cell walls of the ripe fruit, which were probably created by the action of the cell wall degrading enzymes. However, it is important to note that the holes may also be an artefact of the fruit tissue preparation process. During excision of fresh, ripe tissue, the cells may separate readily at the middle lamella by the leverage action of the blade, and possibly tear where the walls are held together at the pit fields, leaving holes in the cells on the surface of the excised tissue.

After deep-freezing at -20℃ the cellular structures of both unripe and ripe fruit appeared damaged (Figures 2.1.3a and 2.1.3b) and the cells seemed to lose their ordered arrangement. The freezing conditions may have caused solidification of oil and crystallization of water in the cells which may have acted as a driving force for cell shrinkage. Rupturing of cell walls due to the formation of large ice crystals with sharp edges (Belitz *et al*., 2004[b]), may have caused the apparent loss of uniform cellular structure after freezing.

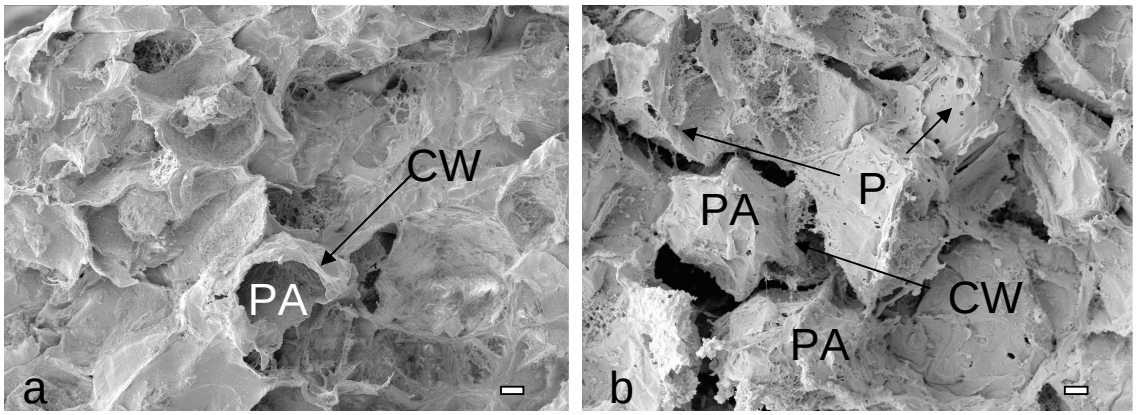


Figure 2.1.3: Scanning electron micrographs of unripe (a) and ripe (b) avocado mesocarp after deep freezing at -20℃ showing parenchyma cells (PA), cell walls (CW) and pits (P). Note partial separation of parenchyma cells in ripe fruit sample (b). Bar represents 10 µm.

Although the middle lamellae were not visible on the micrographs, this structure usually binds adjacent cells together (Mauseth, 1995) and the cells of the unripe fruit still seemed to be bound together (Figure 2.1.3a), while the cells of the ripe fruit seemed to have partially separated from each other (Figure 2.1.3b). Hydrolytic enzymes, apart from possibly creating the pits in the ripe fruit, could have degraded the middle lamellae, causing the cells to move away from each other during freezing and cell shrinkage (Awad & Lewis, 1980). The structural degradation after ripening led to softening of the fruit which was observed during texture analyses. A force of 97.73 g ($p < 0.01$) was needed to penetrate the ripe avocado, while a force of 2842.31 g ($p < 0.01$) was needed to penetrate an unripe avocado.

Effect of fruit ripeness on oil yield

Table 2.1.1 shows that for all extraction methods and fruit pre-treatments, the avocado oil yield from ripe fruit was higher than from unripe fruit.

Table 2.1.1: Oil yield (g kg^{-1} fruit weight on a dry basis) from avocado fruit after extraction with supercritical carbon dioxide (SC-CO_2) and hexane (HEX)

Raw Material	SC-CO_2	HEX
Unripe freeze dried	588 b[*] (20)[†]	677 d (15)
Unripe oven dried (80℃)	522 a (11)	629 c (5)
Ripe freeze dried	648 c (19)	720 e (5)
Ripe oven dried (80℃)	604 b (13)	707 e (12)

[*]Mean values in any column or row followed by the same letter are not significantly different ($p > 0.05$)

[†]Standard deviation in parenthesis

Ripe fruit (freeze-dried and oven-dried combined) had an average oil yield calculated to be 72 g kg^{-1} (7.2%) and 61 g kg^{-1} (6.1%) greater than unripe fruit for SC-CO_2 and hexane extracts respectively. Lewis, Morris & O'Brien, (1978) and Ozdemir and Topuz (2004) reported no significant increase in oil content of *Fuerte* between harvesting and ripening. However, when Lewis *et al*. (1978) analysed the residual oil recovered by saponification of extracted residues, the remaining unripe material seemed to contain slightly more residual oil. The observed increase in oil yield during ripening may be related to changes in the avocado mesocarp at the ultrastructural level. The hydrolytic actions of enzymes such as cellulase and polygalacturonase may bring about degradation of the parenchyma cell walls (Reymond & Phaff, 1965; Zauberman & Schiffmann-Nadel, 1972; Awad & Lewis, 1980). This is shown in the scanning electron micrographs by the presence of pits or small holes in the parenchyma cell walls of the ripe avocado mesocarp (Figure 2.1.2b) which are absent in the unripe avocado mesocarp (Figure 2.1.2a). Such structural degradation could cause the oil to become more available for extraction. In addition to this, phospholipids from the phospholipid bilayer (Garret & Grisham, 1995) might become dislodged and add to the increase in oil yield (Lewis, Morris & O'Brien, 1978).

Effect of method of fruit drying on oil yield

For both unripe and ripe fruit and for both extraction methods, oil yield was higher from freeze-dried material compared to oven-dried (Table 2.1.1). The average oil yield for freeze-dried samples (unripe and ripe combined) was calculated to be 55 g kg^{-1} (5.5%) and 31 g kg^{-1} (3.1%) greater than for oven-dried samples for SC-CO_2 and hexane extracts respectively. However these differences were less for the ripe fruit extracted with hexane where the oil yield from freeze-dried material was not significantly higher than from oven-dried (Table 2.1.1). It is difficult to relate these observations to the ultrastructure of the freeze-dried and oven-dried avocado mesocarp. For unripe and ripe fruit, the cells of the freeze-dried (Figure 2.1.4) and oven-dried (Figure 2.1.5) mesocarp were irregularly shaped and appeared flattened and compact.

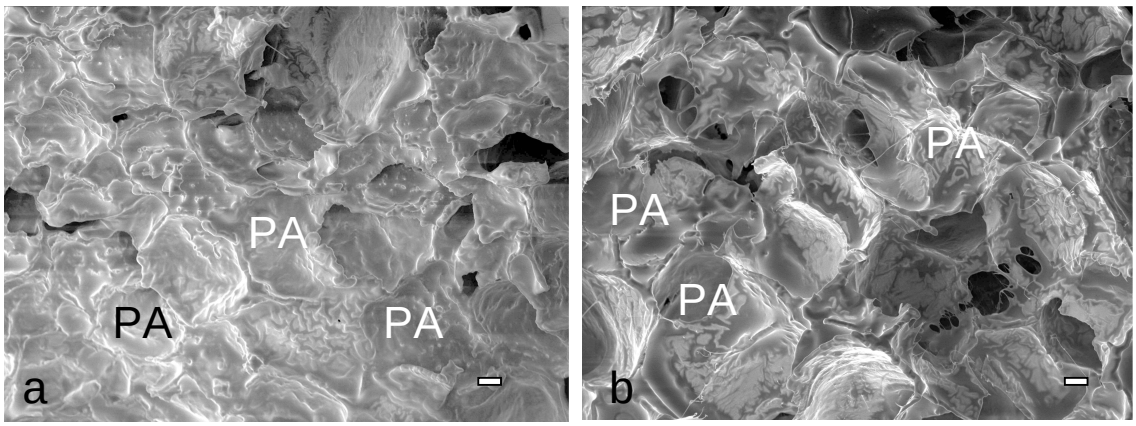


Figure 2.1.4: Scanning electron micrographs of unripe (a) and ripe (b) avocado mesocarp after freeze-drying showing irregularly shaped and flattened parenchyma cells (PA). Bar represents 10 µm.

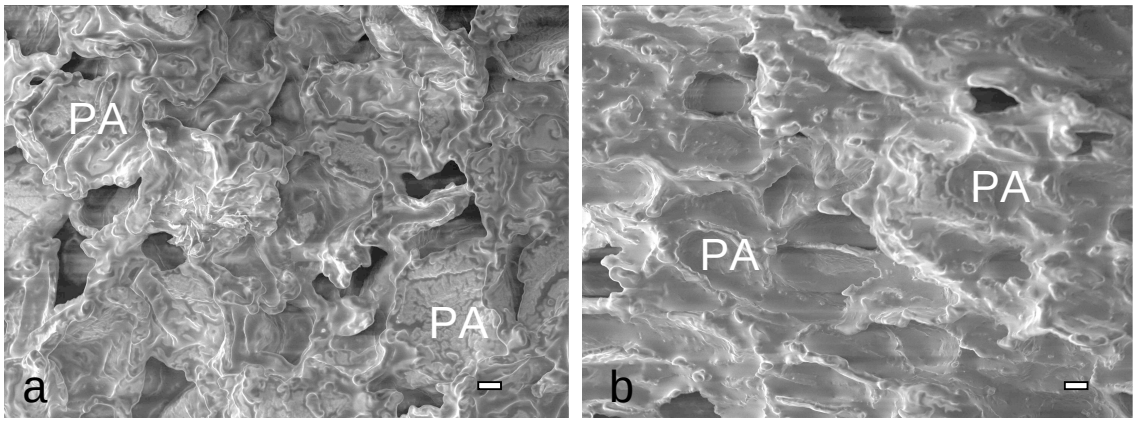


Figure 2.1.5: Scanning electron micrographs of unripe (a) and ripe (b) avocado mesocarp after oven-drying at 80℃ showing irregula rly shaped and flattened parenchyma cells (PA). Bar represents 10 µm.

During oven-drying, denaturing and cross-linking of proteins, and gelatinization followed by dehydration of starch, may lead to formation of a physical barrier around the oil cells (Moreno *et al.,* 2003). This could cause an increase of the mass transfer resistance between the surface and the SC-CO_2 or hexane, thus reducing oil yield obtained after extraction of oven-dried fruit (Gómez *et al*., 1996). The

hardened structure caused by oven-drying could have caused the majority of particles to have a particle size closer to 2 mm while the freeze-dried material, being more brittle, could have been more powdery with the majority of the particles being smaller than those of the oven-dried material. Extraction of smaller particles leads to higher oil yields (Bhattacharjee *et al*., 2007) due to increased surface area.

It must be noted that both oven-dried and freeze-dried fruit samples were pre-frozen mechanically at –20^{o}C before the respective drying processes. The freezing process would be expected to cause the oil to solidify and stay within the cells probably in the form of crystals. As mentioned earlier (Figure 2.1.3), the slow freezing process could also form large ice crystals with sharp edges, damage the cellular structure (Belitz *et al*., 2004^{b}; Gómez *et al*., 1996) and presumably make oil more extractable. During oven-drying, the application of heat would bring about thawing, during which cells do not regain their original shape and turgidity but instead, the cell tissue is softened and cellular material may leak out (Fellows, 1990). Slow freezing and thawing has been shown to cause structural damage to cells of strawberry fruit (Delgado & Rubiolo, 2005).

The heat would also reduce the viscosity of the oil and cause it to leak out to the surface of the fruit particles, resulting in higher oil losses during oven-drying. In contrast, the freeze-drying process involves sublimation of the ice crystals and the relatively lower temperatures used will make melting and leaking of oil much less likely. It is possible that had the oven-dried fruit not been pre-frozen (or perhaps quick frozen in liquid nitrogen), there could have been reduced cell structural damage and oil losses leading to improved oil yields from oven-dried fruit.

Hexane vs. supercritical carbon dioxide extraction

In this study, the oil yield obtained from hexane extraction was significantly higher (with average oil yield calculated as 93 g kg^{-1} (9.3%) greater) than that obtained from SC-CO_2 for all samples (Table 2.1.1). This is in agreement with other reports from literature (Friederich & List, 1982; Gómez & de la Ossa, 2002; Bravi *et al*., 2007). As an extraction solvent, hexane seems to be more indiscriminate and less selective

than CO_2 (Gómez & de la Ossa, 2002; Bhattacharjee *et al*., 2007). Hexane-extracted oils contain relatively higher amounts of non-triglyceride substances such as phospholipids, gums, waxes and contents of the unsaponifiable fraction including sterols, pigments and hydrocarbons (Przybylski *et al*., 1998; Du Plessis, 1980). SC-CO_2-extracted oil is essentially equivalent to a degummed, hexane-extracted crude oil (Gómez & de la Ossa, 2002; Friederich & List, 1982). This could explain the relatively higher yield of hexane-extracted oil.

The SEM micrographs of hexane-extracted samples of both unripe (Figure 2.1.6) and ripe (Figure 2.1.8) fruit (freeze-dried and oven-dried), showed predominantly empty parenchyma cells. In contrast, after extraction with SC-CO_2, unripe (Figure 2.1.7) and ripe (Figure 2.1.9) fruit generally seemed to have a compact, disrupted cellular appearance with some spaces in certain areas of the plant material and relatively fewer empty parenchyma cells were visible. This may be suggestive of relatively more exhaustive and complete oil extraction with hexane compared to SC-CO_2. It may be hypothesised that the SC-CO_2 creates paths of least resistance through the plant material where the SC-CO_2 moves through at greater speeds and volumes (Bhattacharjee *et al*., 2007). The rest of the plant material is resultantly compacted (Figures 2.1.7 & 2.1.9), possibly due to the high pressure at which the extraction is conducted, making it more difficult for the CO_2 to move through these areas. Consequently extraction is not complete in these areas. When all the oil is removed from areas where the CO_2 moves through with the least resistance, the rate of extraction then decreases dramatically. Hexane on the other hand, may percolate and permeate indiscriminately through the whole plant material leading to more exhaustive extraction and greater oil yield.

For the current research extraction with SC-CO_2 was regarded as exhaustive when a yield of less than one percent oil per hour was obtained. A comparison of the quality as well as oxidative stability of the oil extracted with both methods is needed to determine whether the environmental advantage as well as the possible elimination of the de-gumming step of oil extracted with SC-CO_2 (Friederich, & List, 1982) is enough to make this process appealing to the industry.

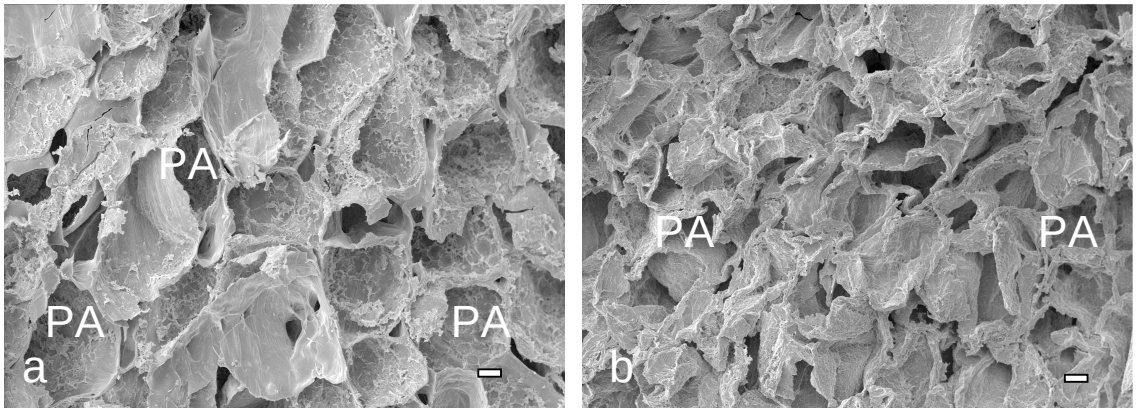


Figure 2.1.6 Scanning electron micrographs of unripe freeze-dried (a) and unripe oven-dried (b) avocado mesocarp after hexane extraction. Note the presence of empty parenchyma cells (PA) throughout the plant material. Bar represents 10 µm.

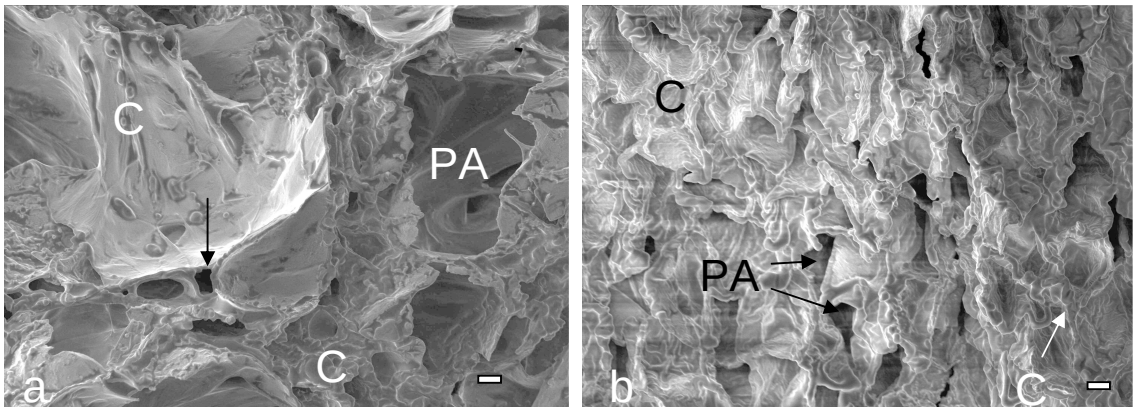


Figure 2.1.7: Scanning electron micrographs of unripe freeze-dried (a) and unripe oven-dried (b) avocado mesocarp after supercritical carbon dioxide (SC-CO_2) extraction. Note compacted areas (C) and loose areas with empty parenchyma cells (PA). Bar represents 10 µm.

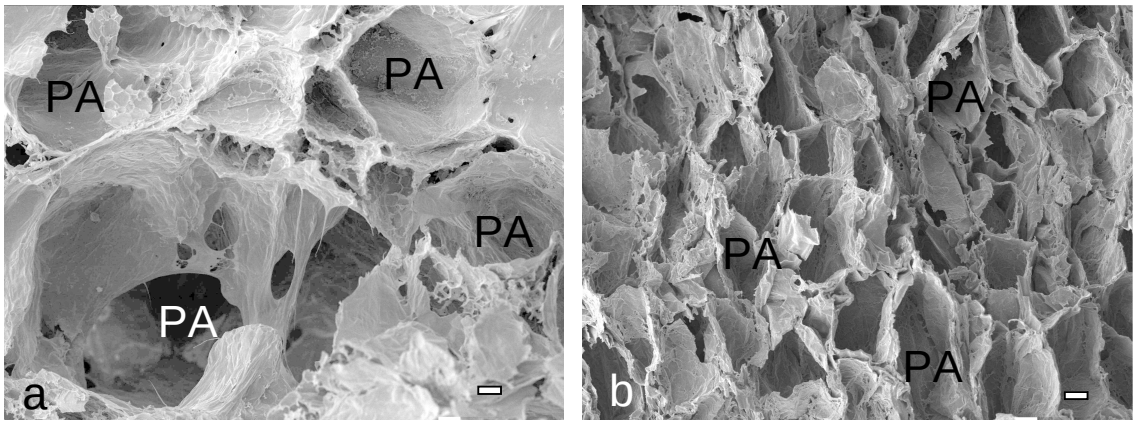


Figure 2.1.8: Scanning electron micrographs of ripe freeze-dried (a) and ripe oven-dried (b) avocado mesocarp after hexane extraction. PA – empty parenchyma cells. Bar represents 10 µm.

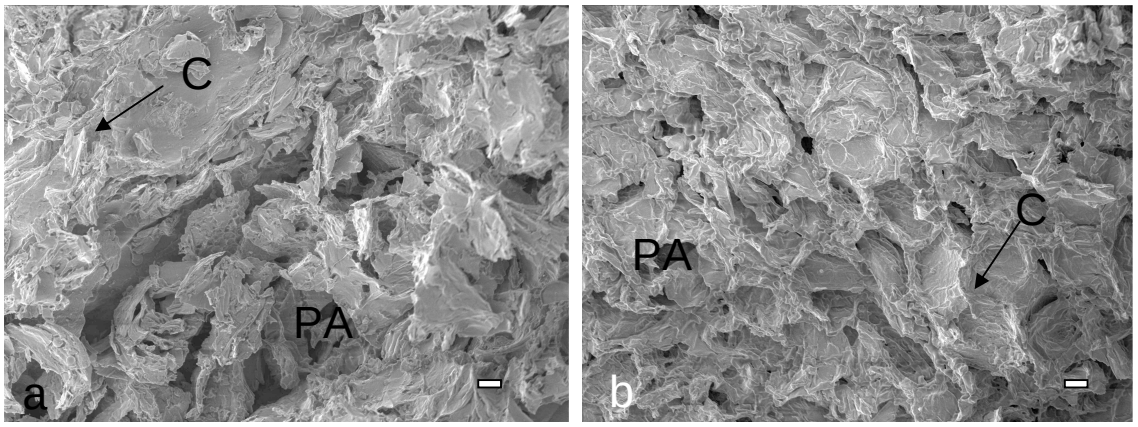


Figure 2.1.9: Scanning electron micrographs of ripe freeze-dried (a) and ripe oven-dried (b) avocado mesocarp after supercritical carbon dioxide (SC-CO_2) extraction. C – compacted areas; PA – empty parenchyma cells in loose areas. Bar represents 10 µm.

2.1.4 Conclusion

Avocado oil yield from ripe fruit is greater than from unripe fruit. This is possibly due to the action of hydrolytic enzymes such as cellulases and polygalacturonase which degrade parenchyma cell walls during fruit ripening thus making the oil more available for extraction. Oil from freeze-dried avocado fruit is more extractable than from oven-dried. This may be due to increased porosity and less structural change within the freeze-dried material compared to formation of structures from changes in protein and starch during oven-drying that act as physical barriers around oil cells. The use of hexane for avocado oil extraction results in higher oil yields than SC-CO_2, possibly because hexane is less selective than SC-CO_2 during extraction. High pressures during SC-CO_2 extraction may result in compacting of the plant material with creation of paths of least resistance within the plant material. Hexane on the other hand, permeates the whole plant material leading to more exhaustive extraction and higher oil yield. In terms of economic feasibility and consumer acceptability it is important to investigate the oil quality obtained after the different pre-treatments as well as hexane and SC-CO_2 extraction.

2.1.5 Acknowledgements

Mr Chris van der Merwe and Mr Allan Hall of the Laboratory for Electron Microscopy and Microanalysis, University of Pretoria are gratefully acknowledged for technical assistance with light and scanning electron microscopy.

2.2 EFFECT OF FRUIT RIPENESS AND METHOD OF FRUIT DRYING ON THE OXIDATIVE STABILITY OF AVOCADO OIL EXTRACTED WITH SUPERCRITICAL CARBON DIOXIDE

ABSTRACT

Supercritical carbon dioxide (SC-CO_2) was used to extract avocado oil from ripe and unripe avocado fruit which had been either freeze-dried or oven-dried (80^{o}C). For all treatments, oil was divided into four fractions and analysed for Fatty acid composition, Peroxide value (PV), Anisidine value (AV), Free Fatty Acids (FFA) and Oxidative Stability Index (OSI). Ripeness and method of drying of the fruit had no effects on the fatty acid composition of the oil. Oil from oven-dried avocado had lower PVs but higher AVs than oil from freeze-dried fruit. FFA levels decreased with progressive extraction, with oil from ripe, freeze-dried fruit having the highest values. OSI increased with progressive extraction (1.08 – 3.04 h in the first fractions to 9.28 – 21.55 h in the fourth). Oil from oven-dried fruit had relatively higher OSI. The OSI correlated positively with oleic acid and negatively with linoleic and linolenic acid. For oil from freeze-dried fruit, FFA correlated negatively with OSI and contributed the most in predicting its OSI. For oil from oven-dried fruit, AV correlated positively with OSI and contributed the most in predicting its OSI.

2.2.1 Introduction

Lipid oxidation is one of the major causes of food spoilage and is of great economic concern to the food industry, as it leads to the development of off-odours and decreases the nutritional value of food (Nawar, 1985). Generally, reaction with molecular oxygen and subjection to elevated temperatures are the main factors influencing the oxidative deterioration of lipids. Other factors include unsaturation and chain length of lipids, packaging, metal ions, moisture, light and antioxidants (Belitz *et al*., 2004[a]). The combinations of moisture and heat as well as enzymes such as lipases are the cause of hydrolytic rancidity (Belitz *et al*., 2004[a]).

Extraction methods and pre-treatment have a significant influence on the oxidative stability and quality of avocado oil produced. Traditional hexane extraction as well as screw pressing requires the avocado fruit to be dry (Southwell, Harris & Swetman, 1990) and the high temperatures and extended drying periods often have a negative influence on oxidative stability. Centrifugal extraction methods usually require the addition of water (Werman & Neeman, 1987; Benedito *et al*., 2004) and the avocado fruit are often overripe, which increases the potential for hydrolytic rancidity.

Supercritical carbon dioxide (SC-CO_2) is gaining in importance in its application for extraction of oils (Bhattacharjee *et al*., 2007) and has been used to extract avocado oil (Botha & McCrindle, 2003). Due to the insoluble nature of phospholipids in SC-CO_2, oil extracted with SC-CO_2 usually has a lower oxidative stability than oils extracted with conventional solvent or screw press methods (List & Friedrich, 1989). Przybylski *et al*. (1998) investigated the oxidative stability of canola oil extracted with SC-CO_2. They found that the oxidative stability of the four canola oil fractions increased with progressive extraction. This was due to the relatively higher content of polyunsaturated fatty acids and free fatty acids (in general) in the first oil fractions which made these fractions comparatively less oxidatively stable than the later fractions.

The aim of this study was to determine the oxidative stability of fractions of avocado oil extracted with SC-CO_2 and how this is influenced by ripeness and pre-treatment of the avocado fruit.

2.2.2 Materials and Methods

2.2.2.1 Preparation of avocado fruit for oil extraction

Horticulturally mature avocado fruit (variety *Fuerte*) was obtained from farms in the Soutpansberg area, Limpopo province, South Africa. This was the same batch of fruit used in Chapter 2.1, page 38. The fruit was divided into two batches. One batch was allowed to ripen and soften by storing at 23℃ for 7 days. The unripe batch was destoned and mechanically cut into pieces of approximately 10 x 10 x 10 mm using a CELME electric slicer (Tracazzano, Italy). The ripened fruit was also cut into pieces of approximately 10 x 10 x 10 mm, using a knife, exercising caution to prevent the formation of a pulp. The unripe and ripe fruit pieces were stored at -20℃ until oil extraction.

Batches of the unripe and ripe fruit pieces were freeze-dried (Virtis Genesis, SP Industries, Warminster, PA, USA) or oven-dried (Forced draft oven, model 361, Labotec, Johannesburg, South Africa) at 80℃ for 24 h to a moisture content of 2-3%. The dried samples were stored at -20℃ until required for oil extraction. Prior to oil extraction, the dried fruit pieces were ground to a particle size < 2 mm in diameter using a Kenwood food processor Model PFP 32 (Shanghai, China). Figure 2.2.1 is a summary of the experimental design for avocado fruit pre-treatment, avocado oil extraction and oxidative stability tests.

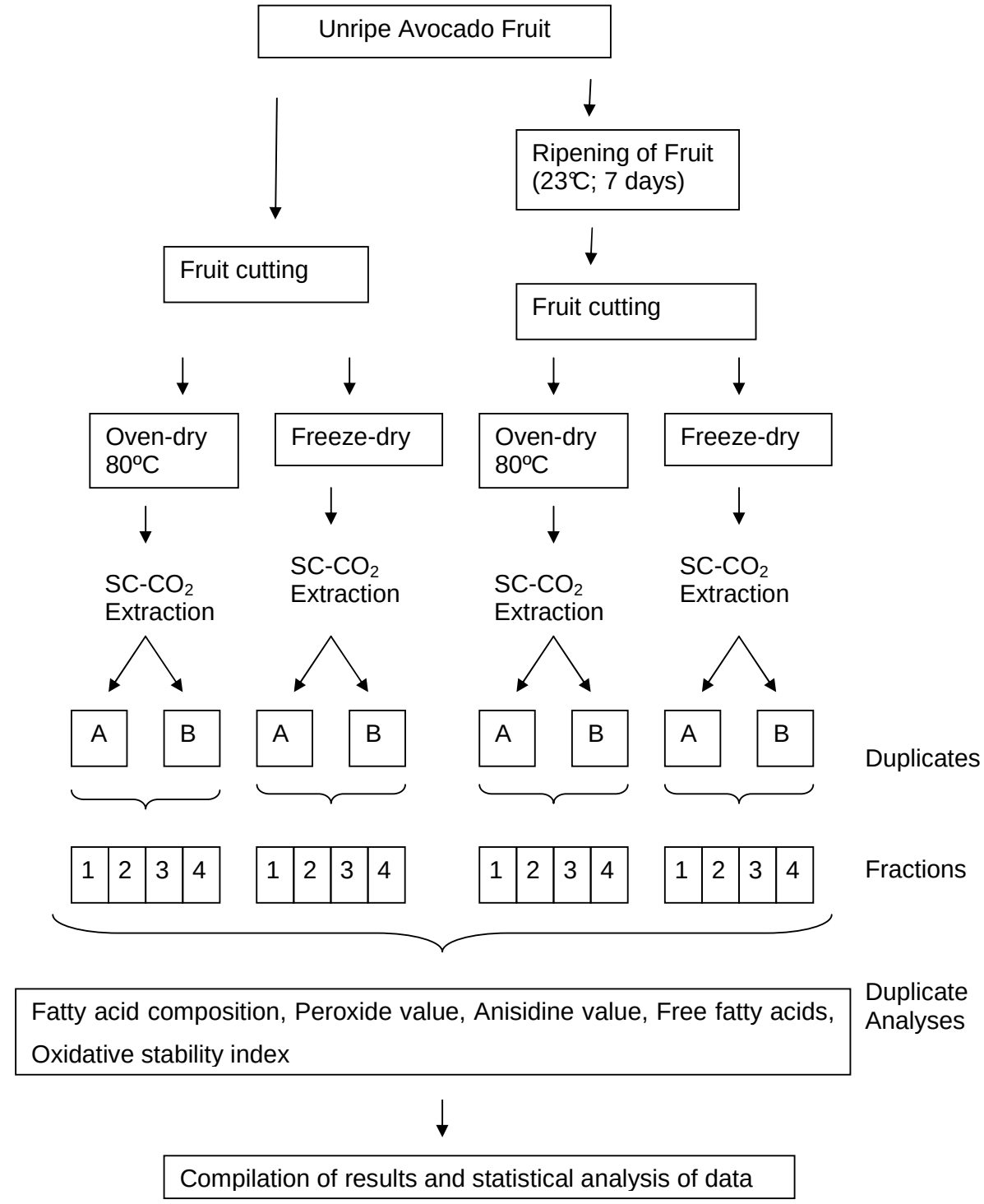


Figure 2.2.1: Summary of experimental design for avocado fruit pre-treatment, avocado oil extraction and oxidative stability tests.

2.2.2.2 Supercritical carbon dioxide (SC-CO_2) extraction of avocado oil

Liquid carbon dioxide, purity 90.0% (Affrox, Johannesburg, South Africa) was used without any further purification, as the extraction fluid. An in-house built apparatus consisting of a 5 l pre-heating column, 10 l extraction vessel and two 5 l separation vessels was used for extraction of 4 kg dried avocado in duplicate. The system was operated by recirculation of the CO_2. The extraction was conducted at 350 atm and 45℃. The conditions in the 2 separators were as follows: separator 1, 100 atm, 40℃; separator 2, 70 atm, 40℃. The flow rate of the CO_2 was measured after step 1 (where a large percentage of the extracted material was already removed from the CO_2 stream) at an average of 60 l/h. Oil fractions were collected every 30 min and extractions were terminated after an oil yield of less than 0.5% (w/w of dry material) was obtained in 30 min. Extractions were conducted under low light conditions and low room temperature of 22^{o}C.

The industrial nature of the plant causes the generation of heat in high pressure pumps which leads to variation in flow rate, consequently influencing the consistency of the extraction rates. This resulted in fractions of avocado oil extracted every 30 min to differ in volume between duplicate extractions as well as treatments. Smaller oil fractions were combined sequentially to reduce it to a total of four fractions per treatment in such a way that the proportions of oil in each of the four fractions were comparatively the same between treatments (Figure 2.2.2).

Oil extractions yielded 30-36 g and 51-57 g oil per 100 g dry mass of avocado pieces for unripe and ripe fruit respectively. Fractions 1 - 4 contained an average of 21, 31, 31 and 17 % respectively of the total amount of oil and represent oil portions obtained with progressive extraction (Figure 2.2.2). These four combined fractions from the different treatments were stored in amber bottles under nitrogen atmosphere at -20^{o}C until analyzed for fatty acid composition and oxidative stability. All analyses were performed under low light conditions.

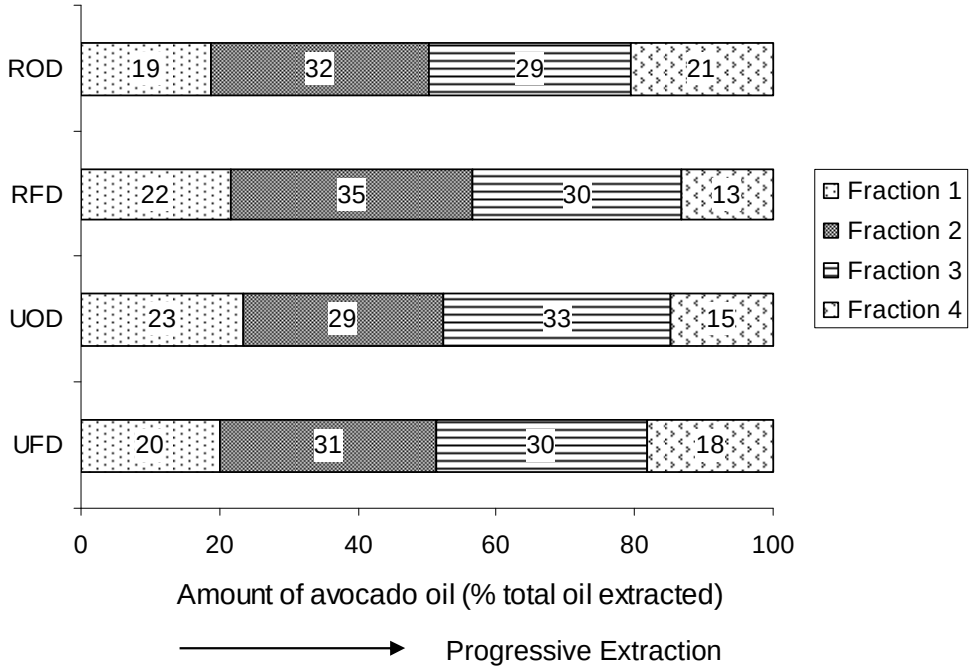


Figure 2.2.2: Division of avocado oil into four consecutive fractions during progressive extraction with supercritical carbon dioxide (SC-CO_2).

* Numbers in the blocks represent the amount of oil in each fraction expressed as a percentage of the total oil obtained over the course of the extraction
ROD – ripe, oven-dried; RFD – ripe, freeze-dried; UOD – unripe, oven-dried; UFD – unripe, freeze-dried

2.2.2.3 Fatty acid composition

The determination of the fatty acid composition procedure was based on the Official Methods and Recommended Practices of the AOCS Method Ce 2-66 (AOCS, 1997) by preparing methyl esters which are separated and determined by capillary Gas Chromatography with Flame ionization detection. A 14% BF_3-methanol (Sigma Aldrich, Munich, Germany) reagent was used for the derivatisation after transesterification with 0.5 M NaOH (Sigma Aldrich, Munich, Germany), in methanol (Sigma Aldrich, Munich, Germany). Three drops of oil sample were derivatised and taken up in 2 mL HPLC grade heptane (Sigma Aldrich, Munich, Germany). One µl of the prepared sample was injected onto an Omegawax^{TM} 320 fused silica capillary

30m column, 0.32 mm ID and 0.25 µm film thickness (Supelco, Johannesburg, South Africa). The oven temperature program was 140℃ for 5 min, after which it was increased to 240℃ at 4 ℃/min and held at 250° C for 10 min. The injector was set at 300℃ and the detector at 240℃. The fatty a cids were expressed as g/100 g total fatty acids. An external fatty acid methyl ester mixture (Supelco 37 Component FAMe Mix 10 000 µg/ml in CH_2Cl_2) was used to identify the fatty acids (47885-U, Supelco, Bellefonte, USA).

2.2.2.4 Oxidative Stability Tests

2.2.2.4.1 Determination of peroxide value (PV), anisidine value (AV) and TOTOX value

The method used for the PV was the AOCS Method Cd 8-53, (AOCS, 1997). Primary oxidation products were measured in terms of milliequivalents of peroxide per kg sample. This was achieved by the addition of potassium iodide which is oxidised to iodine by the peroxides present in the oil. The iodine was measured by titration with standard sodium thiosulphate (Merck, Darmstadt, Germany).

The AOCS Method Cd 18-90 (AOCS, 1997) was used for determination of AV. *p*-Anisidine was re-crystallised according to the method and stored in the dark before use. The method is based on the reaction of aldehydic compounds (principally 2-alkenals and 2,4-dienals) in the avocado oil with *p*-anisidine, in the presence of acetic acid, to form yellowish reaction products which is spectrophotometrically determined at 350 nm. The intensity of the yellowish compounds is not related only to the amount of aldehydic compounds present, but also to their structure. A double bond in the carbon chain conjugated with the carbonyl double bond increases the molar absorbance four to five times. This is why 2-alkenals and dienals will contribute substantially to the value.

The TOTOX value was calculated from the peroxide value and the *p*-anisidine value with the formula 2PV +AV (Hamilton, 1994).

2.2.2.4.2 Determination of free fatty acid value

The AOCS Method Ca 5a-40, (AOCS, 1997) was used with the modification that the oil was dissolved in 80 ML toluene/isopropanol (1:1). The method determines the amount of free fatty acids present in the oil by dissolving the oil sample in a solvent and neutralising it by titration with standard sodium hydroxide, using phenolphthalein as indicator. The results are expressed as g /100 g oleic acid, as oleic acid is the main fatty acid present in most plant oils.

2.2.2.4.3 Oxidative stability index (OSI)

OSI was determined according to the AOCS Method Cd 12b-92 (AOCS, 1997) using a Rancimat 679 instrument (Metrohm Ltd, Herisau, Switzerland). Sample (5 g) was weighed and air was bubbled through at a rate of 20 l/h. The temperature used to conduct the test for the oil samples was 110℃. This was because monounsaturated oils such as palm-olein, avocado and olive oil are much more stable than polyunsaturated oils such as sunflower seed oil and are therefore normally subjected to higher temperature to ensure an induction period less than 48 h, which is the normal maximum run time for the Rancimat instrument. The time from the start of the accelerated stability test to the point at which rapid oxidation occurs is called the induction period and gives an indication of the relative oxidative stability of the oil or fat. The sample is heated in a thermostated heater, while bubbling purified air through it at a constant rate. The effluent air is passed through deionised water and the conductivity of the water is measured for polar oxidation products (mainly formic acid) by an electrode.

2.2.2.5 Statistical Analyses

Statistical analysis was done using ANOVA (Analysis of Variance) as well as multiple regression on STATISTICA ® Kernel release 6M 2006 Edition, StatSoft Inc., Tulsa, USA.

In this research, the data points within each group were not sufficient to determine whether the data was distributed normally. To compensate for this, all residuals,

(the difference between the actual values and the average of the two values in each group), were analysed for normal distribution. The majority of these residuals were normally distributed when tested with the K-S d, Lilliefors and Shapiro-Wilk tests (Lilliefors, 1967; Shapiro, Wilk & Chen, 1968), with the Shapiro-Wilk proving to be the most sensitive. Figure 2.2.3 illustrates the normality of the data distribution as found for the values obtained with the Anisidine value analysis. The *F* test, which is remarkably robust to deviations from normality and where the skewness of the distribution usually does not have a sizable effect on the *F* statistic (Lindman, 1974; Box & Anderson, 1955) was used in the ANOVA to distinguish between the means at a significance level of $p < 0.05$.

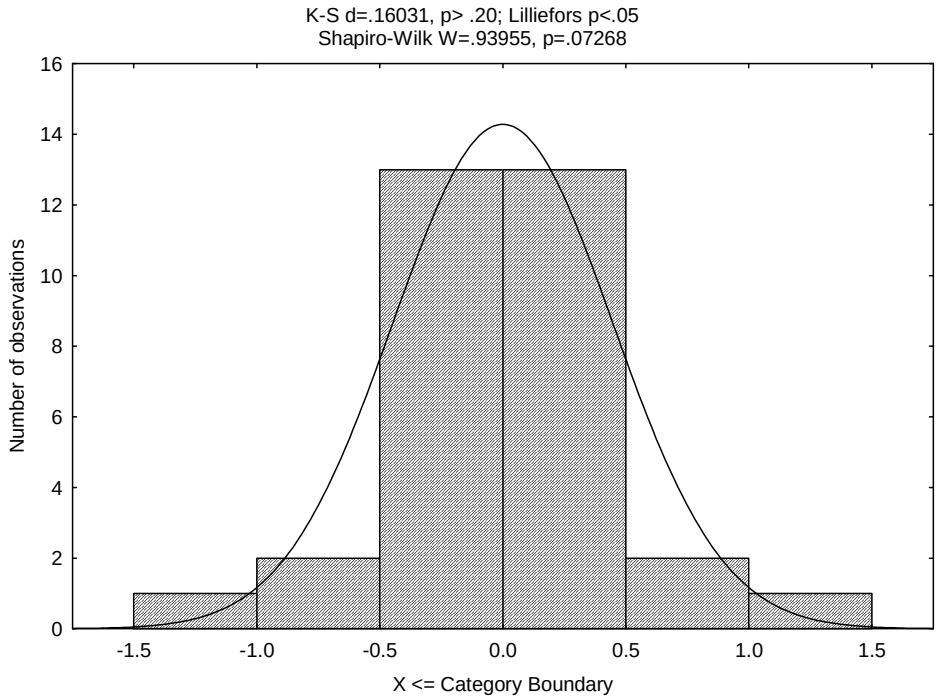


Figure 2.2.3 Histogram displaying the normality of the data as observed from plotting the residuals for all the values obtained for the Anisidine value. Results for the test for normality were as follows: K-S d = 0.16031, $p > 0.20$; Lilliefors $p > 0.05$; Shapiro-Wilk W = 0.93955, p = 0.7268. This histogram is representative for all oxidative tests performed on avocado oil extracted with supercritical carbon dioxide (SC-CO_2).

Multiple regression was performed where the dependent variable was the Oxidative Stability Index (OSI) and the independent variables were the values obtained for the various oxidative stability tests (PV, AV, FFA) for the different fractions and treatments. Relevant variables were selected with forward stepwise regression where independent variables were individually added or deleted from the model at each step until the model with the best fit was obtained. The *F to enter* value, which determines how significant the contribution of any particular variable has to be in order to be added to the equation, was set at 2.0.

2.2.3 Results and Discussion

2.2.3.1 Fatty acid composition

The fatty acid profile represents the macro-component fraction of oils and together with glycerol, is also referred to as the saponifiable fraction (Beltiz, Grosch & Schieberle, 2004[a]). The fatty acid composition provides valuable information regarding the stability of the oil. Oils with higher levels of unsaturation are associated with lower levels of oxidative stability.

The fatty acid profile of the *Fuerte* avocado oil analysed in this study is given in Table 2.2.1. The main fatty acid was oleic acid (C18:1) (71.20 – 77.17%), followed by palmitic (C16:0) (10.20 – 11.39 %) and linoleic acid (C18:2) (7.12 – 7.61 %). Levels smaller than 0.1 g/ 100 g fatty acids were obtained for C15:0 (isopalmitic) and C17:0 (margiric acid) (results not shown). The fatty acid profile of oil extracted from avocado fruit varies according to which stage of the season it was harvested (Du Plessis, 1980; Poiana *et al*., 1999). Avocado oil is classified as monounsaturated oil with oleic acid being the dominant fatty acid ranging from 58% early in season to 80% later in the season for *Fuerte* (Du Plessis, 1980; Ratovohery, Lozano & Gaydou, 1988).

Table 2.2.1: The fatty acid composition (g/ 100 g fatty acids) of avocado oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2)

		C16:0	C16:1	C18:0	C18:1n9	C18:2n6	C18:3n3	C20:1	C20:3n6	C21:0	C22:2	C24:1n9
UFD	**1**	10.84 a	2.75 de	0.62 a	71.27 a	7.29 defg	0.45 a	0.17 a	0.29 a	0.18 a	0.24 a	0.48 c
	2	11.01 a	2.70 cde	0.66 ab	74.44 bcde	7.38 defg	0.45 a	0.16 a	0.12 a	Trace	0.12 a	0.24 abc
	3	10.85 a	2.52 bcde	0.67 ab	75.31 de	7.19 abc	0.44 a	0.19 a	0.11 a	Trace	Trace	0.22 ab
	4	10.99 a	2.54 bcde	0.71 ab	74.17 cde	7.23 bcde	0.45 a	0.19 a	0.10 a	Trace	Trace	0.23 abc
UOD	**1**	11.39 a	2.77 de	0.77 ab	73.18 ab	7.41 efg	0.45 a	0.15 a	0.20 a	0.11 a	0.15 a	0.29 abc
	2	10.95 a	2.59 bcde	0.71 ab	74.41 bcde	7.34 cdefg	0.45 a	0.17 a	0.18 a	0.10 a	0.13 a	0.29 abc
	3	10.68 a	2.38 abc	0.74 ab	75.91 ef	7.19 ab	0.43 a	0.19 a	0.12 a	Trace	Trace	0.20 ab
	4	10.49 a	2.34 ab	0.76 ab	75.95 ef	7.20 abc	0.44 a	0.19 a	0.12 a	Trace	Trace	0.22 ab
RFD	**1**	10.84 a	2.62 bcde	0.65 ab	71.45 a	7.40 fgh	0.45 a	0.09 a	0.16 a	0.27 a	0.17 a	0.44 bc
	2	11.17 a	2.73 cde	0.66 ab	74.01 bcd	7.61 h	0.48 a	0.17 a	0.15 a	Trace	0.13 a	0.31 abc
	3	10.78 a	2.40 abc	0.73 ab	76.09 ef	7.35 bcdefg	0.45a	0.19 a	Trace	Trace	Trace	0.18 a
	4	10.20 a	2.12 a	0.83 b	77.17 f	7.12 a	0.44 a	0.23 a	Trace	Trace	Trace	0.11 a
ROD	**1**	11.19 a	2.84 e	0.63 a	71.30 a	7.43 gh	0.47 a	0.17 a	0.30 a	0.15 a	0.16 a	0.34 abc
	2	11.17 a	2.78 de	0.65 ab	73.46 bc	7.59 h	0.48 a	0.18 a	0.19 a	Trace	0.12 a	0.27 abc
	3	10.74 a	2.48 abcd	0.72 ab	75.34 de	7.33 bcdef	0.46 a	0.20 a	0.15 a	Trace	Trace	0.19 ab
	4	10.59 a	2.35 abcd	0.77 ab	75.76 def	7.21 abcd	0.44 a	0.21 a	0.15 a	Trace	Trace	0.17 abc
Pooled STDEV		0.22	0.06	0.03	0.25	0.03	0.00	0.03	0.06	0.04	0.03	0.04

¶Mean values in the same column followed by the same letter are not significantly different ($p > 0.05$)

UFD – unripe, freeze-dried; UOD – unripe, oven-dried; RFD – ripe, freeze-dried; ROD – ripe, oven-dried

Trace values represent values < 0.10 g/ 100 g fatty acids

The degree of ripeness and drying method of the fruit did not influence the levels of the individual fatty acids relative to each other and it can therefore be concluded that these variables did not have a significant effect on the fatty acid profile of avocado oil extracted with SC-CO_2.

Przybylski *et al*. (1998) reported that triglycerides with unsaturated fatty acids were extracted more efficiently at the beginning of extraction of canola oil with SC-CO_2. The contribution of linolenic and linoleic acids were 20 and 10% higher in the first fraction than the last. They also found that the levels of saturated and monounsaturated fatty acids increased with progressive extraction. This same trend was also observed by other authors (Snyder, Friederich, & Christianson, 1984; Fattori, Bulley & Meisen, 1987; Favati, King, & Mazzati, 1991).

For all treatments, the first two oil fractions had higher levels of C18:2 than the last two. Although they occurred at very low levels in the oil, C20:3 and C22:2 were higher in the first two fractions and lower or in trace amounts in the last two. These results indicate a decreasing trend in the levels of polyunsaturated fatty acids in the avocado oil with progressive extraction, in agreement with other authors (Snyder *et al*., 1984; Fattori *et al*., 1987; Favati *et al*., 1991; Przybylski *et al*., 1998). There was however, no significant change in the levels of C18:3 with progressive extraction. For the saturated and monounsaturated fatty acids, the levels of C18:1 increased with progressive extraction. However, a slight decrease in the levels of C16:1 and C24:1 was observed. No significant changes were observed in the levels of C16:0, C18:0, C20:1 and C21:0 with progressive extraction.

2.2.3.2 Peroxide value, anisidine value and TOTOX value

Since the peroxide value is always interpreted better in combination with the anisidine value, it is important to evaluate these two values together. The PVs (Figure 2.2.4) and the AVs (Figure 2.2.5) of the avocado oil samples did not follow a specific consistent trend with progressive oil extraction.

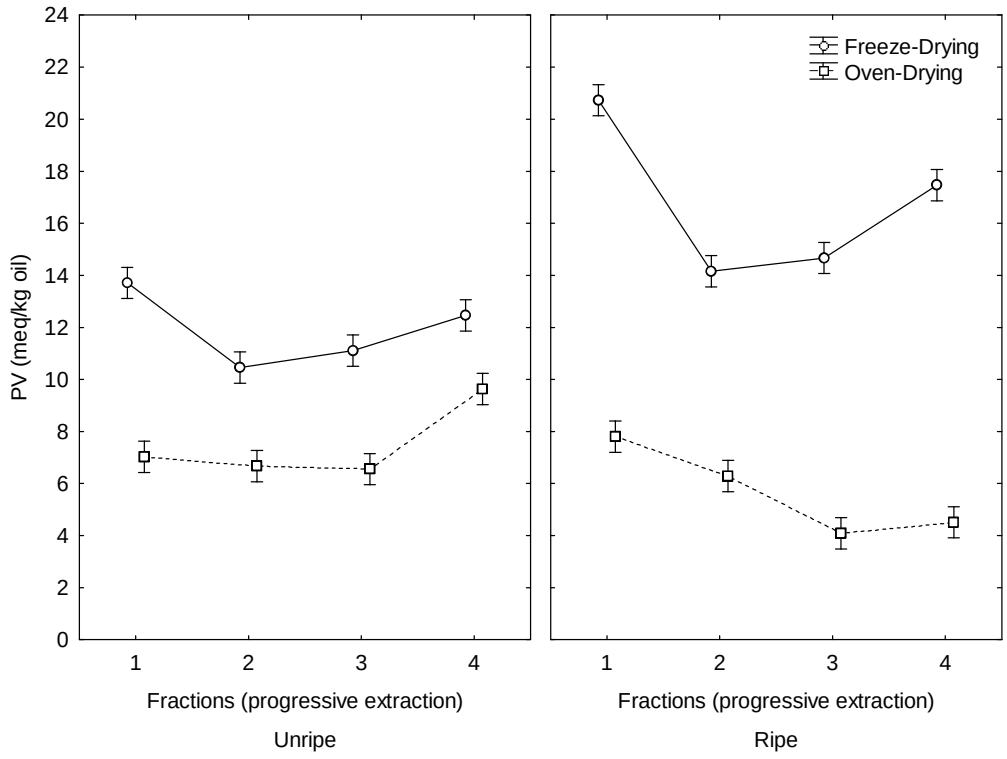


Figure 2.2.4: Peroxide value of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

Of more significance were the effects of fruit pre-treatment. For both unripe and ripe fruit, oil extracted from oven-dried avocado had lower PVs (4.10 – 9.64 meq/kg) than oil extracted from freeze-dried fruit (10.46 – 20.73 meq/kg) (Figure 2.2.4) but higher AVs (16.04 – 35.97 mmol/kg) than oil from freeze-dried fruit (2.63 – 6.31 mmol/kg) (Figure 2.2.5).

Heat is one of the most important aggravators of oxidative rancidity (Belitz *et a*l., 2004[a]). After prolonged heating during oven-drying, hydroperoxides, measured by the peroxide value, are broken down to secondary oxidation products, like aldehydes, which are measured by the anisidine value (Hamilton, 1994).

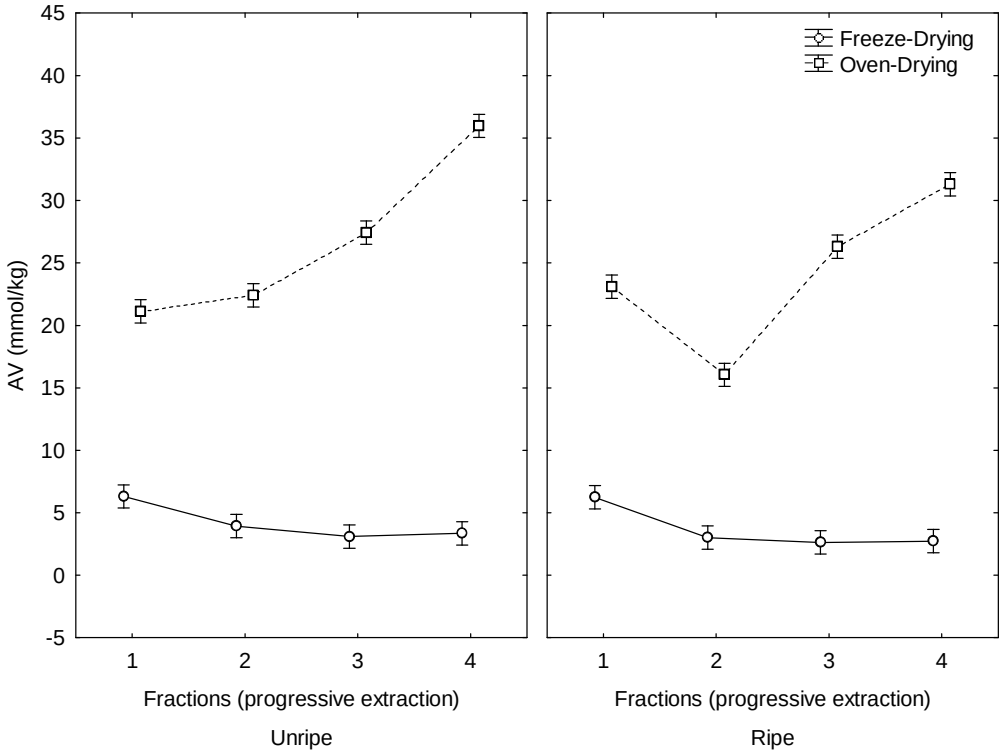


Figure 2.2.5: Anisidine value of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

This would account for the low PVs but high AVs for oil from oven-dried avocado. On the other hand, relatively lower drying temperatures during freeze-drying of avocado could lead to fewer aldehydes formed from hydroperoxides, resulting in high PVs but low AVs for oil from freeze-dried fruit.

Differences in PV between oil from unripe fruit compared to ripe were more evident in the freeze-dried samples. The PV range of oil extracted from ripe freeze-dried fruit was higher (14.15 – 20.73 meq/kg oil) than that of oil extracted from unripe freeze-dried fruit (10.46 – 13.71 meq/kg oil). Ripe fruit has higher lipoxygenase content than unripe fruit (Pesis, Fuchs & Zauberman, 1978; Platt & Thomson, 1992). Higher lipoxygenase activity would lead to increased oxidation of unsaturated fatty acids,

resulting in higher formation of hydroperoxides and higher PVs in oil from ripe, freeze-dried fruit.

Freshly refined oil should preferably have a PV below 1 meq/kg, but values of up to 10 meq/kg are allowed (White, 1995). Sensory rancidity is perceived at values above 10 meq/kg (Rossel, 1994). Both refined and crude oils can have an AV ranging between 1 and 10 mmol/kg (White, 1995; Crapiste *et al*., 1999).

Oil samples from freeze-dried fruit had PVs at or above 10 meq/kg (Figure 2.2.4). Though PVs of oil from oven-dried fruit were below this threshold value, their corresponding AVs were high (Figure 2.2.5). This indicates that oil samples from freeze-dried fruit were at a point where significant production of secondary oxidation products was beginning to occur. On the other hand, the relatively lower peroxide values of oil from oven-dried fruit suggests that it had already reached the threshold point of 10 meq/kg oil and the sufficient production of secondary oxidation products that lead to a rancid odour, had occurred. These observations suggest that the rate of oxidation was higher in oil samples from oven-dried fruit and are a further indication that heating speeds up the oil oxidation process (Sanders, 1994).

The TOTOX value is often considered useful in that it combines evidence about the past history of the oil, in the AV, with that of the present state of the oil, in the PV (Hamilton, 1994). Figure 2.2.6 shows the calculated TOTOX values of oil extracted from unripe and ripe avocado fruit. From their relative positions on the graphs, it is clear that the values were generally highest in oil from unripe, oven-dried fruit and lowest for unripe freeze-dried fruit. Values for oil from ripe fruit were intermediate. The mean TOTOX value for both ripe and unripe fruit was higher for oven-dried fruit (38.58) than for freeze-dried fruit (32.61). These results indicate that in general, the oil from oven-dried fruit had undergone higher total oxidative deterioration than oil from freeze-dried fruit and are in agreement with the observations from the PV and AV results above.

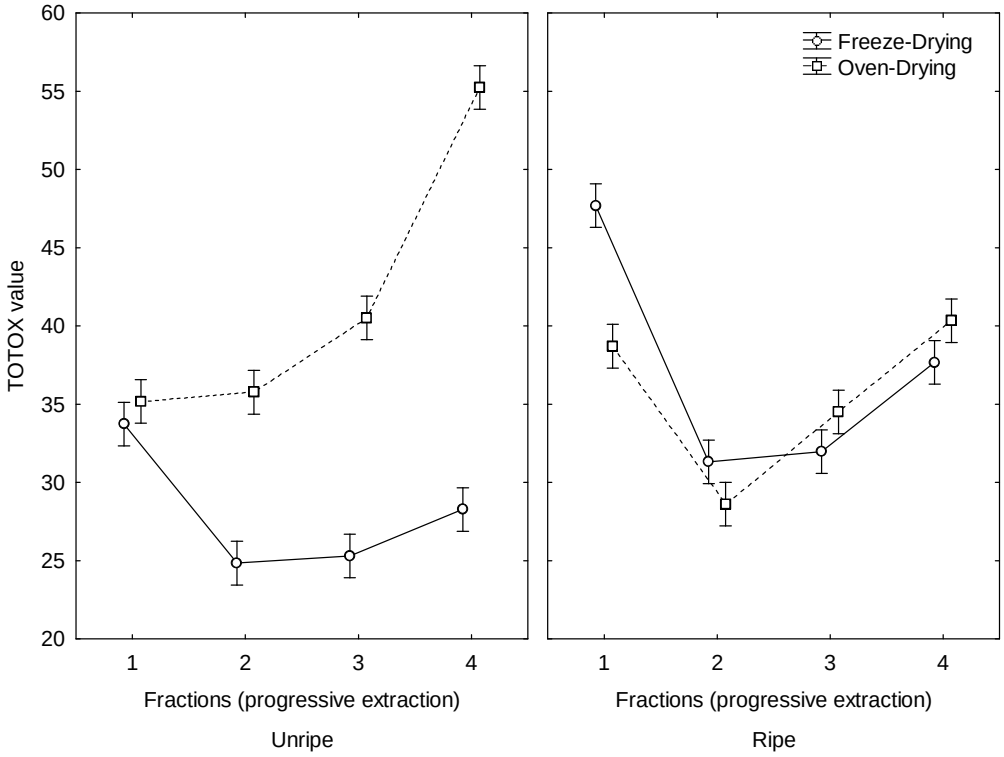


Figure 2.2.6: TOTOX value of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

2.2.3.3 Free Fatty Acids

The free fatty acid value of oil is usually an indicator of hydrolytic deterioration (Rossell, 1994). Free fatty acids produced by hydrolytic degradation are preferred by lipoxygenase as substrates (Hamilton, 1994) leading to oxidative deterioration.

The free fatty acid content of the avocado oil ranged between 0.47 and 2.15 g oleic acid/100 g oil (Figure 2.2.7). According to the Codex Alimentarius Commision (1999), FFA values for refined oils should be below 0.3% (0.3 g oleic acid/100 g oil), while crude oils can reach up to 5% (5 g oleic acid/100 g oil), depending on the oil type. Therefore the FFA values of the avocado oil were well within the prescribed limits. There was generally a decreasing trend in FFA levels with progressive extraction for oil from all fruit pre-treatments. This was more pronounced for oil from

ripe, freeze-dried fruit. Przybylski *et al*. (1998) also reported higher levels of free fatty acids in the first fractions of canola oil extracted with SC-CO_2 compared to later fractions. For the first two fractions, FFA values of oil extracted from ripe, freeze-dried fruit were significantly higher than those of oil from the other treatments. The total level of free fatty acids for all four fractions combined was highest for oil from ripe, freeze-dried fruit (4.91 g oleic acid/100 g oil) compared to the other treatments (3.24, 3.73 and 2.31 g oleic acid/100 g oil for oil from unripe freeze-dried, unripe oven-dried and ripe oven-dried fruit respectively). Ripening of climacteric fruit is associated with an increase in the levels of various enzymes (Prasanna *et al*., 2007) and it is possible that the preservation of lipase activity under the low temperature conditions of freeze-drying compared to oven-drying, could have led to greater release of free fatty acids in ripe, freeze-dried fruit compared to the other treatments.

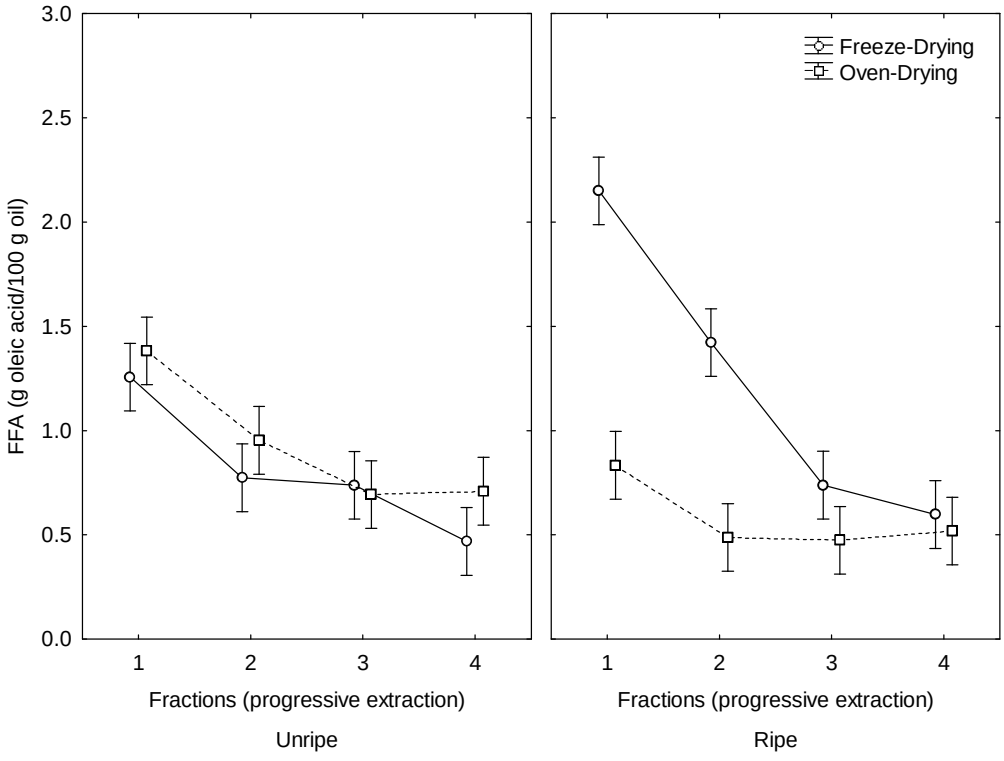


Figure 2.2.7: Free fatty acid content of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2) Vertical bars denote 0.95 confidence intervals.

2.2.3.4 Oxidative stability index (OSI)

Although the OSI is an accelerated oxidative stability test, it seems to be one of the best indicators of the shelf life of oil (Van der Merwe, 2003). It is especially useful in ranking oils of the same type according to their oxidative stability (Rossell, 1994). Figure 2.2.8 shows that the OSI of the avocado oil increased with progressive extraction. This was in accordance with Przybylski *et al*. (1998), who reported that the oxidative stability of canola oil increased during progressive extraction with SC-CO_2. The values obtained for the first two fractions of oil extracted from ripe and unripe freeze-dried fruit (1.08 – 5.95 h) were comparable to those reported by Poiana *et al*. (1999) (3 – 5 h) for crude oil extracted by centrifugal extraction from ripe avocados (*Bacon*, *Hass* and *Reed*).

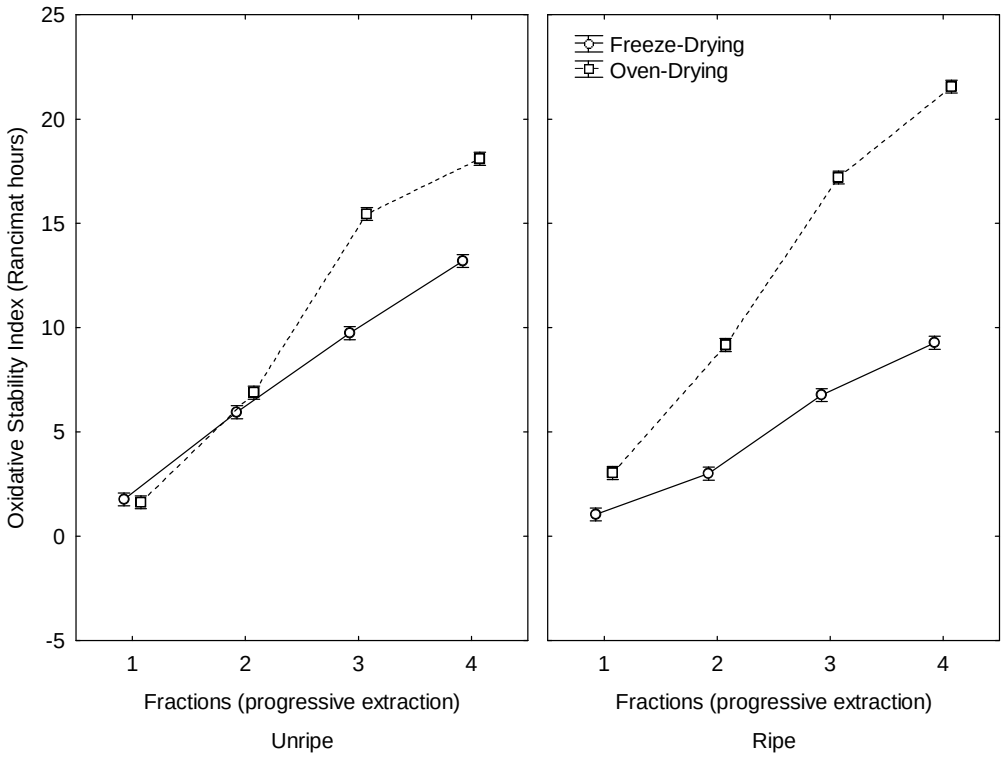


Figure 2.2.8: Oxidative stability index (Rancimat hours) of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

According to Rossell (1994), oils with an induction period of 9 h can be classified as moderately stable. Oil from ripe oven-dried fruit, had already reached this point in the second fraction (9.17 h). Oil from unripe oven-dried fruit reached this threshold somewhere between the second and third fraction (6.89 – 15.45 h) while oil from unripe freeze-dried fruit acquired this status in the third fraction (9.74) followed by oil from ripe freeze-dried fruit, only reaching the threshold in the fourth and final fraction (9.28). According to the above classification, oil from ripe oven-dried fruit had the highest oxidative stability, while oil from ripe freeze-dried fruit had the lowest oxidative stability. In summary, oil from the oven-dried fruit samples had higher oxidative stability than oil from freeze-dried samples. These observations appear to be at odds with the results obtained for the TOTOX values which indicated the opposite. A possible explanation for this could be the formation of compounds with antioxidant activity during the heat treatment of the oven-dried samples which confer high oxidative stability to the oil.

2.2.3.5 Correlations

The OSI correlated positively with oleic acid (0.68) and negatively with linoleic (-0.53) and linolenic (-0.42) acid. Being a polyunsaturated fatty acid, linoleic acid will oxidise more rapidly than monounsaturated or saturated fatty acids (Sonntag, 1979[b]). A higher content of linoleic acid relative to the content of more saturated fatty acids, would therefore lead to a lower induction period on the OSI. In the same way, higher oleic acid content relative to the polyunsaturated fatty acids, would lead to a longer induction period on the OSI. The main saturated fatty acid present in the avocado oil, palmitic acid (C16:0), correlated negatively with the OSI (-0.49). This was not expected, as a higher content of saturated fatty acids should indicate a higher OSI (Sonntag, 1979[b]). A negative correlation was also observed between C16:1 and the OSI. The correlations between the fatty acids alone did not sufficiently explain the results obtained from the OSI.

The high negative correlation (-0.74) between the peroxide value and anisidine value of avocado oil from both freeze-dried and oven-dried fruit (Table 2.2.2A) was expected as a decrease in the primary oxidation products measured by the PV is usually accompanied by an increase in secondary oxidation products measured by the AV (Rossell, 1994).

A positive correlation between the TOTOX value and the AV (0.60, Table 2.2.2A) was also expected as high total oxidation values indicate that oxidation has progressed to such an extent that a high amount of secondary products have been produced.

There was a positive correlation (0.53) between the FFA and PV of avocado oil from both freeze-dried and oven-dried fruit (Table 2.2.2A). High levels of free fatty acids could lead to the production of more hydroperoxides (Rossell, 1994) through oxidation and could increase the PV of the oil. The negative correlation (-0.69) between FFA and the OSI (Table 2.2.2A) indicates that a high FFA content would lead to a low OSI. High levels of free fatty acids, especially if unsaturated, would make the oil prone to oxidative deterioration which implies a decrease in OSI. The positive correlation between the AV and OSI (Table 2.2.2A) is anomalous and cannot be explained by oil oxidation laws. However, separate correlation matrices for oil from freeze-dried (Table 2.2.2B) and oven-dried fruit (Table 2.2.2C) provide some insight.

Table 2.2.2B shows that for oil from freeze-dried fruit, AV (-0.74) and FFA (-0.84) had significant negative correlations with the OSI. According to Rossell (1994), high AV and FFA values would lead to oil with a low oxidative stability. This fact is reinforced by these correlations for oil extracted from freeze-dried fruit. Oil extracted from freeze-dried fruit, therefore portray expected correlations indicating that the formation of the hypothesized antioxidant did not occur during freeze-drying.

Table 2.2.2: Correlation coefficients between peroxide value (PV), anisidine value (AV), TOTOX value, percentage free fatty acids (FFA) and the oxidative stability index (OSI) of avocado oil extracted with supercritical carbon dioxide (SC-CO_2) for (A) both freeze-dried and oven-dried fruit, (B) freeze-dried fruit and (C) oven-dried fruit.

A	PV	AV	TOTOX	FFA	OSI
PV	1.00	-0.74***	0.10	0.53**	-0.50**
AV	-0.74***	1.00	0.60***	-0.23	0.56**
TOTOX	0.10	0.60***	1.00	0.29	0.23
FFA	0.53**	-0.23	0.29	1.00	-0.69***
OSI	-0.50**	0.56**	0.23	-0.69***	1.00

B	PV	AV	TOTOX	FFA	OSI
PV	1.00	0.36	0.98***	0.63*	-0.44
AV	0.36	1.00	0.53*	0.74**	-0.74**
TOTOX	0.98***	0.53*	1.00	0.72**	-0.55*
FFA	0.63*	0.74**	0.72**	1.00	-0.84***
OSI	-0.44	-0.74**	-0.55*	-0.84***	1.00

C	PV	AV	TOTOX	FFA	OSI
PV	1.00	0.19	0.62*	0.40	-0.31
AV	0.19	1.00	0.89***	-0.24	0.73**
TOTOX	0.62*	0.89***	1.00	-0.01	0.44
FFA	0.40	-0.24	-0.01	1.00	-0.73**
OSI	-0.31	0.73**	0.44	-0.73**	1.00

*,**,*** indicate significance at $p < 0.05$, 0.01 and 0.001, respectively.

As observed for oil from freeze-dried fruit (Table 2.2.2B), there was a significant negative correlation (-0.73) between the FFA and OSI for oil from oven-dried fruit (Table 2.2.2C). However, there was a significant positive correlation (0.73) between AV and OSI for oil from oven-dried fruit (Table 2.2.2C) in contrast to what was observed for oil from freeze-dried fruit (Table 2.2.2B). As suggested before, a possible explanation for the positive correlation between AV and OSI for oil from oven-dried fruit could be the formation of compounds with antioxidant activity as a result of the heating during oven-drying of the fruit prior to oil extraction. Predictive models developed for the OSI of the oil seem to support such a theory.

2.2.3.6 Models

Predictive models (Table 2.2.3) were developed to determine the effect of PV, AV and FFA (independent variables) on the OSI (dependent variable) of avocado oil extracted with SC-CO_2. Model 1 was developed using the three independent variables for oil extracted from oven-dried and freeze-dried fruit together. Model 2 was developed using the data obtained from oil extracted from freeze-dried fruit. Model 3 was developed with the same variables from data obtained from oil extracted from oven-dried fruit. The predicted versus observed values are shown in Figure 2.2.9.

Model 1 indicated that the FFA value (which was selected first), on its own, was the best predictor of the OSI of avocado oil extracted with SC-CO_2 compared to the PV and AV. It also had the highest β-value in magnitude (-0.75), showing that it made the largest contribution to the OSI value. The negative sign of the β-value of the FFA indicates that it had a negative influence on the OSI, which agrees with the negative correlation between the FFA value and the OSI for oil from freeze-dried and oven-dried fruit together (Table 2.2.2A).

Table 2.2.3: Statistical parameters obtained from regression of oxidative stability index (OSI) of avocado oil extracted with supercritical carbon dioxide (SC-CO_2) as dependent variable and peroxide value (PV), anisidine value (AV) and free fatty acids (FFA) as independent variables for the three models

N = 31 cases	β* (Beta) regression coefficients	B regression coefficients	Standard error of B coefficients	p-level§
Model 1 (Oil from freeze-dried and oven-dried fruit)				
Intercept		7.11	3.25	0.037
FFA	-0.75	-10.44	1.77	0.000
AV	0.68	0.37	0.09	0.000
PV	0.41	0.55	0.25	0.035
¶R^2 = 0.71; ♥F (3,28) = 22.6; ⌜Standard error of estimate = 3.62				
N = 15 cases				
Model 2 (Oil from freeze-dried fruit)				
Intercept		11.75	3.25	0.004
PV	0.14	0.18	0.24	0.481
AV	-0.16	-0.44	0.59	0.472
FFA	-0.82	-6.11	1.88	0.007
R^2 = 0.73; F (3,12) = 10.93; Standard error of estimate = 2.39				
N = 15 cases				
Model 3 (Oil from oven-dried fruit)				
Intercept		7.02	3.51	0.069
PV	-0.26	-1.07	0.40	0.020
AV	0.67	0.80	0.11	0.000
FFA	-0.47	-11.43	2.37	0.000
R^2 = 0.92; F (3,12) = 44.28; Standard error of estimate = 2.32				

*β (Beta): Coefficients derived from standardized data. Standardized data were derived by dividing the experimental values by the standard deviation for that variable. Beta values give an indication of the relative importance of the different components.

§p-level: resulting probability value from t- and F-tests. It indicates the significance of the values obtained.

¶R^2: square of the correlation coefficient. It measures the degree of association between the dependant and the independent variables and an indicator of how well the model fits the data (e.g. an R^2 close to 1.0 indicates almost all of the variability with the variables specified in the model have been accounted for).

♥F: tests the significance of the relationship between the dependant variable and the set of independent variables. In this case the numerator was calculated to have 9 (10 regression coefficients – 1) degrees of freedom and the denominator had 21 (31 cases – 10 regression coefficients) degrees of freedom.

⌜Standard error of estimate: measurement of the dispersion of the observed values about the regression line.

The magnitude of the β-values for model 1 also show that after FFA, the AV followed by the PV were the next important variables in predicting the OSI. Both PV and AV had positive regression coefficients according to model 1, indicating that they had a positive influence on the OSI. The positive regression coefficient for AV corresponds with the positive correlation between AV and OSI (Table 2.2.2A). This may be regarded as an anomaly because an increase in the AV usually indicates an increase in secondary oxidation products in the oil and will also mean that the OSI will be low (Hamilton, 1994). The positive regression coefficient for PV did not correspond with the negative correlation between PV and the OSI (Table 2.2.2A).

Chemically, the regression coefficient of the PV can be either positive or negative because the PV increases until the hydroperoxides start breaking down to secondary products, where after it will decrease again (Rossell, 1994). The sign of the regression coefficient for the PV would depend on the state of oxidation of oil at the point where the PV was measured.

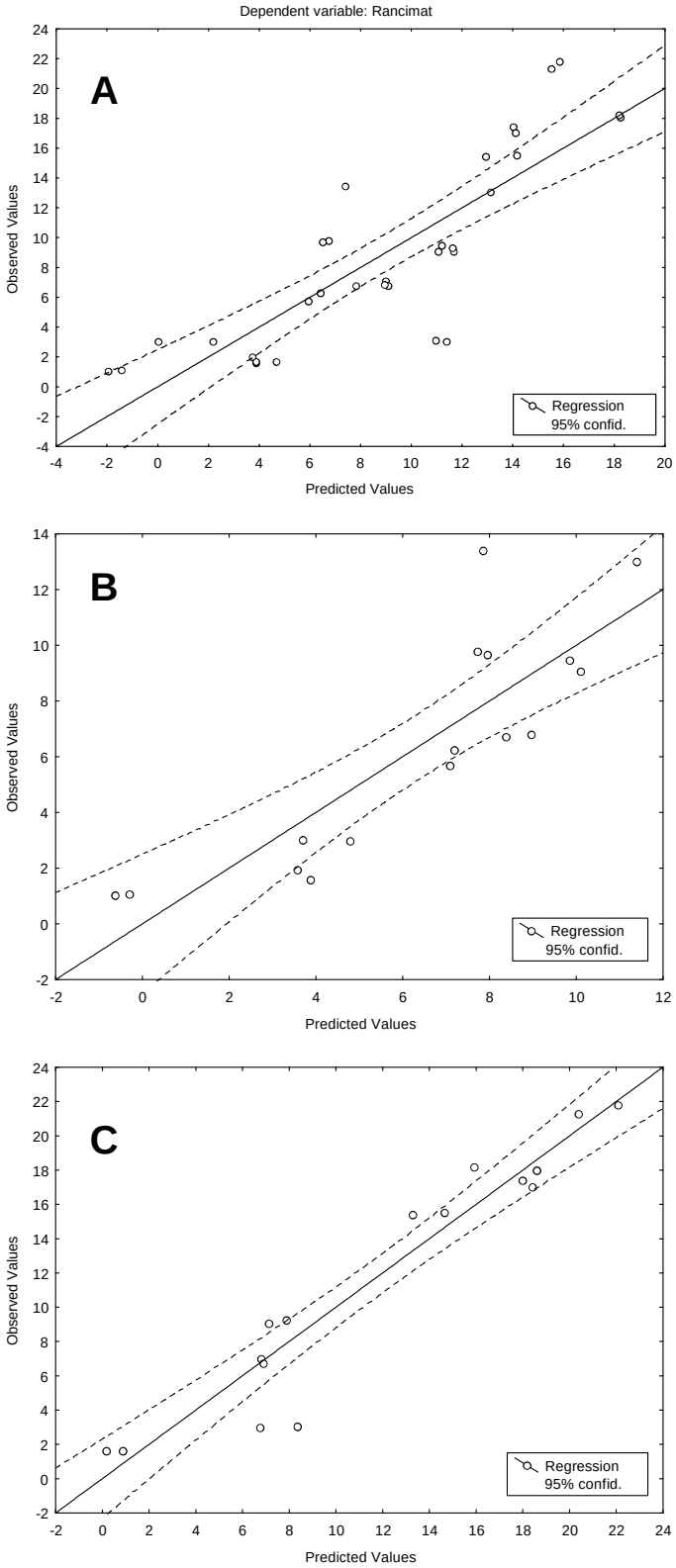


Figure 2.2.9: Predicted versus observed oxidative stability index values for avocado oil extracted with supercritical carbon dioxide (SC-CO_2). A – oil from freeze-dried and oven-dried fruit; B – oil from freeze-dried fruit; C – oil from oven-dried fruit.

If the peroxide value was measured while it was increasing, the regression coefficient could be positive, while a negative regression coefficient between PV and OSI would mean that it was measured while it was decreasing. The PVs used for model 1 were for oil from both oven-dried and freeze-dried fruit. It is therefore possible that the PVs of the two oils were perhaps measured at different stages of oxidation which could influence the models.

The order of selection for model 2 (Table 2.2.3) was PV, followed by AV and lastly, FFA. The FFA had the highest β-value in magnitude (-0.82) and therefore the largest contribution to the prediction of the OSI.

The regression coefficient was negative, indicating that the FFA had a negative influence on the OSI, which is in agreement with the chemistry of oil oxidation (Van der Merwe, 2003). The PV and AV had similar contributions to the OSI in terms of magnitude. Both contributions were small and not significant ($p > 0.05$), indicating that only the FFA had a significant contribution to the OSI of avocado oil extracted with SC-CO_2 from freeze-dried fruit. This predictive model indicates that the hydrolytic deterioration in freeze-dried avocado was more significant to the prediction of the OSI than the oxidative deterioration measured by the PV and AV. This could be attributed to the possible preservation of lipase enzymes in the freeze-dried material leading to the hydrolytic degradation of the oil. Unsaturated fatty acids, when released from glycerol are prone to oxidative deterioration leading to a decrease in OSI.

From model 3 (Table 2.2.3) it can be seen that PV was selected first, followed by AV and FFA. The β-values, on the other hand, indicated that the AV had the largest, positive contribution in magnitude to the OSI ($\beta = 0.67$). The AV was followed by the FFA value ($\beta = -0.47$) and PV (-0.26) which both had a negative influence on the OSI.

All of the variables had a significant contribution ($p < 0.05$) to the OSI. On an initial, superficial investigation, this model is not chemically acceptable since an increase in the AV is expected to decrease the OSI (Hudson & Gordon, 1994). This model

corresponded well with the correlation matrix for oil from oven-dried fruit (Table 2.2.2C) where the correlations between PV, AV, FFA and the OSI were: -0.31, 0.73 and -0.73 respectively. The contribution of the FFA to the OSI of oil from oven-dried fruit (model 3) was much less than that of oil from freeze-dried fruit (model 2) probably due to inhibition of enzyme (lipase) activity from exposure to high temperatures during oven-drying (Harris & Tall, 1994). The AV, which is an indication of oxidative deterioration caused by the exposure to heat and oxygen, would be expected to increase under the prolonged heating conditions of oven drying, which could lead to decreased oxidative stability. However, the OSI of oil from oven-dried fruit was high (Figure 2.2.8) notwithstanding its high AV (Figure 2.2.5).

Model 3 therefore shows that the OSI of oil from oven-dried fruit is not negatively compromised by high AV. These observations lend support to the proposed hypothesis that the oven-drying process could have led to formation of some antioxidant compounds. Such antioxidant compounds may exert strong antioxidant effects thus overriding the effect of the AVs, and leading to high OSI.

These models, although not applicable in certain aspects to conventional oil chemistry laws, were applicable in the prediction of the OSI of avocado oil subjected to the pre-treatment and extraction conditions of this study. It seems that even though the oxidative deterioration of the oil, measured by the peroxide and anisidine values was rather advanced (Rossell, 1994), some compounds with high antioxidant activity may have been formed as a result of the heat treatment during oven-drying and these may have exerted strong antioxidant effects particularly in oil from oven-dried fruit. The next chapter examines how changes in micro-components due to pre-treatment may influence the OSI of the oil.

2.2.4 Conclusions

The fatty acid profile of avocado oil extracted with SC-CO_2 is not influenced by the degree of ripeness or drying method of the fruit. Oil extracted from unripe and ripe oven-dried avocado has lower PVs but higher AVs than oil from freeze-dried fruit.

While there is a decrease in levels of FFA in oil samples from all treatments with progressive extraction, oil from ripe freeze-dried fruit have the highest levels of FFA. The OSI of oil samples from all treatments increases with progressive extraction with oil from oven-dried fruit having relatively higher OSI than oil from freeze-dried fruit. Oleic acid correlates positively while linoleic acid correlates negatively with the OSI. There is a significant negative correlation between FFA and OSI and AV and OSI for oil from freeze-dried fruit and from mathematical modelling, the FFA contributes the most to prediction of the OSI of this oil. For oil from oven-dried fruit, correlation between FFA and OSI is negative but positive between AV and OSI and the AV contributes the most to prediction of the OSI. It is hypothesised that the high OSI of oil from oven-dried fruit, notwithstanding its high AV, may be due to the presence of compounds with high antioxidant activity in the oil formed through the heat treatment of the oven-drying process.

2.2.5 Acknowledgements

Dr P. J. Van Niekerk is gratefully acknowledged for invaluable assistance with statistics and advice on some scientific and technical aspects.

2.3 CHARACTERIZATION OF MICRO-COMPONENTS AND DEVELOPMENT OF PREDICTIVE MODELS FOR THE OXIDATIVE STABILITY INDEX OF AVOCADO OIL EXTRACTED WITH SUPERCRITICAL CARBON DIOXIDE

ABSTRACT

Avocado oil was extracted from horticulturally mature ripe and unripe fruit with supercritical carbon dioxide (SC-CO_2). The fruit were either freeze-dried or oven-dried at 80^{o}C prior to oil extraction. For each treatment, the oil was divided into four fractions with progressive extraction. The oil fractions were analysed for colour, chlorophyll, carotenoid, total unsaponifiables, tocopherol and sterol contents and oxidative stability index (OSI). Intensity of blue and red on the Lovibond colour scale of all oil samples increased with progressive extraction. The third (78.04 – 210.03 mg pheophytin/kg) and fourth (249.13 – 394.42 mg pheophytin/kg) oil fractions had higher levels of chlorophyll than the first two fractions (23 – 58 mg pheophytin/kg). There was a progressive increase in carotenoid content from the first (6.67 – 15.53 mg/kg) to the last fractions (34.22 – 70.51 mg/kg). Apart from oil from ripe, oven-dried fruit, oil from the other fruit samples had higher levels of total unsaponifiables in the first than the latter fractions. Levels of total tocopherols (117.9 – 405.8 mg/kg), total sterols (3277.5 – 4373.2 mg/kg) and their isomers did not show any specific trends with progressive extraction. β-Tocopherol was the lowest tocopherol isomer while α-tocopherol was the highest isomer and β-sitosterol was the most abundant sterol isomer. The OSI increased with progressive extraction and correlated positively with chlorophyll (0.83) and carotenoids (0.80). From multiple regression models, chlorophyll, carotenoids and tocopherols have a positive influence on the oxidative stability of avocado oil extracted with SC-CO_2, while total sterols seem to have a negative effect.

2.3.1 Introduction

Avocado oil is important as an edible oil due to its health-enhancing qualities and is especially used in the treatment of connective tissue diseases (Maheu *et al*., 1995). Current methods used for extraction of avocado oil include cold pressing of heat-dried unripe fruit (Carr, 1997), solvent extraction (Owusu-Ansah, 1997) and the centrifugal extraction of ripe fruit (Benedito *et al*., 2004). Supercritical carbon dioxide (SC-CO_2) as an extraction fluid, offers some advantages when compared to conventional solvents. These include their high diffusivity, low viscosity, low surface tension and the ability to speed up mass transfer (Garcia *et al*., 1996). SC-CO_2 has been applied extensively in the extraction of oils from plant sources (Garcia *et al*., 1996; Gómez & de la Ossa, 2002; Botha & McCrindle, 2003). Apart from the report of Botha and McCrindle (2003), there appears to be a lack of information on the extraction of avocado oil with SC-CO_2.

An important quality characteristic of all edible oils is their oxidative stability. This is governed by their content of micro-components in the unsaponifiable fraction that often act as antioxidants (Frega *et al*., 1993). In oil extracted from fresh avocado fruit, the unsaponifiables amount to 1 – 2 % compared to 3 – 7 % in oil extracted from dried avocado fruit (Farines *et al.*, 1995). Micro-components in the unsaponifiable fraction include higher aliphatic alcohols, sterols, tocopherols, carotenoids, other pigments and hydrocarbons (Farines *et al*., 1995). In exerting antioxidant effects, some of these micro-components may function synergistically as has been observed between phospholipids and β-carotene (Woodall, Briton & Jackson, 1997), flavonoid antioxidants and phospholipids (List & Friedrich 1989; Oshima, Fujita & Koizumi, 1993) β-carotene and α-tocopherol (Palozza & Krinsky, 1992) and phospholipids and tocopherol. Although sterols are not specifically known for their antioxidant activity, δ-5 avenasterol, vernosterol, fucosterol and citrostadienol have been shown to have antioxidant activity in oils at 180ºC (Sims *et al*., 1972; White & Armstrong, 1986; Rajalakshmi & Narasimhan, 1995).

In many cases, the decomposition of the antioxidant may yield substances with a pro-oxidant action (Sonntag, 1979[b]). Tocopherols are well-known for their pro-oxidant effect above the optimum concentration for effectiveness as antioxidants (Sonntag, 1979[a]). The most encountered pro-oxidants in fats are trace metals from tanks and other metallic equipment, cultivation soils and pesticides with which the soil or plant was treated during cultivation (Sonntag, 1979[b]). Another inherent substance of moist oil seeds, chlorophyll is a strong pro-oxidant in the presence of light. It does however seem to have antioxidant potential when oil is not exposed to light (Endo *et al*., 1985[a]; Endo *et al*., 1985[b]; Zambiazi, & Przybylski, 1998; Psomiadou & Tsimidou, 2002).

The micro-components contained in avocado oil are affected by factors such as avocado fruit ripeness, fruit pre-treatments prior to extraction of the oil and the extraction process itself. The avocado only ripens after harvesting (Awad & Lewis, 1980; Sippel, 2001; Ozdemir & Topuz, 2004), which occurs at horticultural maturity. Ripening could affect the content and composition of the micro-components in the oil. The application of heat during oven-drying of avocado fruit (generally at 80^{o}C) prior to oil extraction, could affect the functional properties of the micro-components in avocado oil negatively by destroying the phytochemicals contained in the oil. The use of freeze-drying could be expected to minimize or eliminate such losses in functional properties of the phytochemicals. One of the strengths of SC-CO_2 as an extraction fluid is its ability to separate heat-sensitive substances and production of high-purity products (Araujo, *et al*., 2001). It has been used for enrichment of fats and oils to obtain products with improved functionality for specific applications or with better nutritional values (Ibáñez *et al*., 2002), and to produce oils enriched in sterols (Przybylski *et al*., 1998; Chuang & Brunner, 2006) and pigments (Przybylski *et al*., 1998). Enrichment seems to occur during progressive extraction which can also be attributed to the different solubilities of these compounds in SC-CO_2 (Brunetti *et al*., 1989).

This chapter reports on a study of the micro-components of avocado oil extracted with SC-CO_2 from unripe and ripe avocado fruit subjected to oven- or freeze-drying.

The development of predictive models for the oxidative stability index of the avocado oil based on the micro-component content was also attempted.

2.3.2 Materials and Methods

2.3.2.1 Preparation of avocado fruit for oil extraction

The avocado fruit samples were prepared as described in Chapter 2 p. 54.

2.3.2.2 Supercritical carbon dioxide (SC-CO_2) extraction of avocado oil

SC-CO_2 extraction was executed as described in Chapter 2 p. 56. Subsequent analyses on the oil samples were conducted under low light conditions and low room temperature of 22^{o}C.

2.3.2.3 Lovibond colour determination

The AOCS Method Cc 13e-92 (AOCS, 1997) was used. The method is used for colour determination of oil by matching the colour of light transmitted through glass colour standards to the colour of light transmitted through a specific depth of oil, but originating from the same source. The avocado oil was first filtered through Whatman no 1 filter paper (Schleicher & Shuell, Maidstone, UK) to prevent interferences due to any turbidity and read on the Lovibond Tintometer (Colour Laboratory, Salisbury, UK).

2.3.2.4 Determination of chlorophyll pigments

The chlorophyll pigments content was determined by AOCS method Cc 13i-96 (AOCS, 1997). This spectrophotometric method involves measuring the absorbance of the oil sample at 630, 670 and 710 nm in a 10 mm quartz cell. Prior to analyses the oil was filtered through Whatman no 1 filter paper (Schleicher & Shuell, Maidstone, UK) to ensure that the oil was free from any particles. Due to the dark green colour of some of the avocado oil fractions, all samples were diluted (1 g of oil in 10 mL of solvent and the true value calculated by multiplying the anwswer by 10) with spectrophotometric grade hexane (Sigma Aldrich, Munich, Germany),

which was also used in the reference cell. The content of chlorophyll pigments was expressed in mg of pheophytin *a* per kg of oil which was calculated as follows:

$$C = 345.3 \times \frac{[A_{670} - (0.5 \times A_{630}) - (0.5 \times A_{710})]}{L}$$

Where:

C = content of chlorophyll pigments as mg of pheophytin *a* in 1 kg of oil

A = absorbance at the respective wavelength

L = light path of the spectrophotometer cell (mm)

2.3.2.5 Determination of unsaponifiable matter

AOCS method Ca 6a-40 (AOCS, 1997) was used to determine the unsaponifiable matter in avocado oil. This method determines those substances dissolved in oil which cannot be saponified by alkali treatment, but are soluble in ordinary oil solvents and includes higher aliphatic alcohols, sterols, pigments and hydrocarbons. The oil was filtered before analyses through Whatman no 1 filter paper (Schleicher & Shuell, Maidstone, UK) to ensure that the oil was free from any particles which could interfere with the weight of the unsaponifiable fraction. Approximately 5 g of oil was measured out into a round bottomed flask and saponified by boiling under reflux for 1 hr with 30 mL 90 % ethanol (Merck, Darmstadt, Germany) and 5 mL 50% KOH (Sigma Aldrich, Munich, Germany). The saponified mixture was transferred to a separating funnel and the round bottomed flask was washed with approximately 5 mL of 95% ethanol which was transferred to the separating funnel up to the 40 mL mark. A further 40 mL of warm water was used in 10 mL aliquots to wash the flask and added to the separating funnel. After the washing step, the unsaponifiable matter was extracted eight times with 80 mL of petroleum ether (Merck, Darmstadt, Germany). The combined petroleum ether fractions (approximately 640 mL) were washed with distilled water until the wash solution no longer gave a pink colour after the addition of one drop of phenolphthalein solution (1% in 95% ethanol). The petroleum ether extract was evaporated to dryness under vacuum at 40^{o}C using a

Labo Rota S-300 rotary evaporator (Resona Technics, Gossau, Switzerland). The flasks were dried in an oven (60℃) (model 361, Lab otec, South Africa) to constant weight (designated A). The residue was then taken up in 50 mL of warm ethanol (95%) containing phenolphthalein indicator, previously neutralised and titrated with 0.02 N NaOH to a permanent pink colour. The weight of the residue was corrected for free fatty acid content using the relationship 1 mL of 0.02 N NaOH is equivalent to 0.005 g of oleic acid and designated B. The amount of oleic acid from a blank determination was designated C. The amount of unsaponifiable matter was calculated as follows:

$$\text{Unsaponifiable matter (g/kg)} \quad \frac{A - (B + C)}{\text{Mass of sample (g)}} \times 1000$$

2.3.2.6 Determination of total carotenoid content

The Malaysian Palm Oil Board Test Method p2.6:2004 was used to determine the total carotenoid content of the avocado oil. The absorbance of 0.15 g of filtered (Whatman no 1, Schleicher & Shuell, Maidstone, UK) avocado oil dissolved in 25 mL spectrophotometric grade iso-octane (Sigma Aldrich, Munich, Germany) was measured at 446 nm (the maximum UV absorbance for β-carotene in iso-octane) on a Phillips PU 8620 UV/visible spectrophotometer (Phillips, Johannesburg, South Africa). Iso-octane was used in the reference cell. The total carotenoid content was determined by using the following formula:

$$\text{Total carotenoid content (mg/kg)} = \frac{V \times a \times 10\,000}{W \times 2610}$$

Where V = Volume of iso-octane (25 mL)
a = Absorbance at 446 nm
W = Weight of sample taken (g)
2610 = Extinction coefficient of β-carotene in iso-octane

2.3.2.7 Determination of tocopherol content

Tocopherol isomers were measured by normal phase HPLC using a Shimadzu LA 101 system (Shimadzu, Columbia, USA), a C-R8A chromatopac integrator (Shimadzu, Columbia, USA) and a Perkin Elmer LS 30 fluorescence detector with excitation at 295 nm and emission at 330 nm as described by Van Niekerk (1973) and Van Niekerk (1975) and amended as in AOCS Method Ce 8-89 (AOCS, 1997). The amendments were as follows: 0.5 g of avocado oil was made up to 10 mL in the mobile phase (1 % isopropanol in hexane) and filtered into a 10 mL vial using a Target 0.45 µm PTFE filter (Anatech, Johannesburg, South Africa). Separation was obtained by injecting 20 µl of the solution onto a 25 cm Luna NH_2 5 µm 100Å column (Phenomenex, Torrance, USA) and applying a flow rate of 3 mL/min. Because only α -, β – and γ – tocopherol were readily obtainable commercially, a mixture of maize, sunflower and soya oils was used in this study, containing seven of the eight basic compounds of vitamin E (tocopherols and tocotrienols) which were previously identified according to their retention times (Van Niekerk, 1975). Only δ –tocotrienol was not present in the test sample. α -Tocopherol (98 % HPLC, Fluka, Buchs, Switzerland) was used as an external standard. A calibration curve was drawn up using the α -tocopherol and relative response factors were used to determine the concentrations of the other tocopherols and tocotrienols. The detection limit for tocopherols is 0.1 mg/100 g (Van Niekerk, 1975).

2.3.2.8 Determination of sterol content

The ISO/FDIS method 1228 (ISO, 1999) was used for determination of sterols by gas chromatography. Avocado oil (250 mg) and 1 mL internal standard [1.0 mg/mL betulin (97% Sigma Aldrich, Munich, Germany) in HPLC grade acetone (Merck, Darmstadt, Germany)] was measured into a round bottom flask and saponified under reflux with 5 mL 0.5 mol/l ethanolic KOH (Sigma Aldrich, Munich, Germany) for 15 min. After saponification it was immediately diluted with 5 mL 95 % ethanol (Sigma Aldrich, Munich, Germany), swirled to homogenize and 5 mL of the solution was pipetted onto an aluminium oxide column [10 g of aluminium oxide (Merck, Darmstadt, Germany) suspended in 20 mL of ethanol (Merck, Darmstadt, Germany)]. The initial eluent was discarded and the unsaponifiable matter was

eluted with 5 mL of ethanol and then with 30 mL of di-isopropyl ether (Merck, Darmstadt, Germany) at a flow rate of about 2 mL/min. Solvents in the eluates were removed by evaporation under vacuum using a Labo Rota S-300 rotary evaporator at 40℃ (Resona Technics, Gossau, Switzerland) in order to obtain the unsaponifiable matter. This was then dissolved in 0.6 mL of di-isopropyl ether and applied onto a TLC plate (Sigma Aldrich, Munich, Germany). TLC was conducted using 100 mL hexane:di-isopropyl ether [1:1 (V/V)] as the developing solvent with 5 µl cholesterol (1.0 mg/mL) and betulin (5.0 mg/mL) as sterol standards.

The TLC plate was allowed to dry in a fume cupboard and sprayed with methanol to reveal the sterol and betulin spots which appeared white on a dark background. The lines were marked and scraped off into a small beaker and 0.05 mL of ethanol was added. The silica gel in the beaker was digested three times with 5 mL of di-isopropyl ether and filtered (Whatman no 1, Schleicher & Shuell, Maidstone, UK) into a flask. The extracts were dried to about 1 mL in a rotary evaporator, transferred to a 0.3 mL reaction vial with a solid cap and a PTFE liner (Supelco, Bellefonte, USA) and dried with a stream of nitrogen. Silylation reagent (100 µl) [50 µl of 1-methylimidazol (99% GC grade, Sigma Aldrich, Munich, Germany) to 1 mL of N-Methyl-N-trimethylsylyl-heptafluoroburyramide (MSHFBA) (>90% GC grade, Fluka, Buchs, Switzerland) was added to the isolated sterols in the vial, sealed and placed in an oven for 15 min at 105℃. The vial was taken out and left to cool to room temperature, where after it was immediately injected into the capillary gas chromatograph with Flame Ionisation Detection (Varian 3800, Palo Alto, USA). A 30 m SE-54 (Supelco, Bellefonte, USA) capillary column with an internal diameter of 0.25 mm and a film thickness of 0.1 µm was used. The carrier gas was H_2 at a flow rate of 36 cm/s, with a split ratio of 1:20, detector temperature 260℃, injector temperature 270℃ and injection volume of 1 µl. The temperature program was held at 260℃ for 10 min and then increased at a rate of 3℃/min to 280℃.

2.3.2.9 Determination of the oxidative stability index (OSI)

The OSI was determined as described in Chapter 2 p. 59.

2.3.2.10 Statistical analyses

Statistical analysis was done using ANOVA (Analysis of Variance) as well as multiple regression on STATISTICA ® Kernel release 6M 2006 Edition, StatSoft Inc., Tulsa, USA. The *F* test (Lindman, 1974) was used to determine significant differences between means at a significance level of $p < 0.05$.

In similar fashion to what was done in Chapter 2 (P 59), all residuals of the data obtained in this chapter, (the difference between the actual values and the average of the two values in each group), were analysed for normal distribution using the K-S d, Lilliefors and Shapiro-Wilk tests (Lilliefors, 1967; Shapiro *et al*., 1968). Figure 2.3.1 illustrates the normality of the data distribution as found for the values obtained for campesterol.

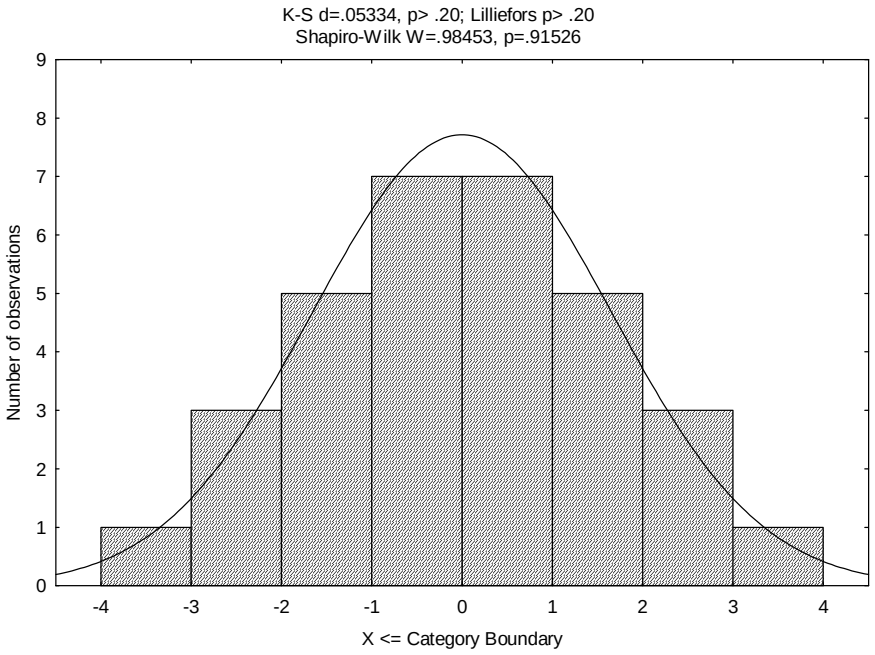


Figure 2.3.1: Histogram displaying the normality of the data as observed from plotting the residuals for all the values obtained for campesterol. Results for the test for normality were as follows: K-S d = 0.05334, p > 0.20; Lilliefors p > 0.20; Shapiro-Wilk W = 0.98453, p = 0.91526. This histogram is representative for all the micro-components analysed in the avocado oil extracted with supercritical carbon dioxide (SC-CO_2).

2.3.3 Results and Discussion

2.3.3.1 Lovibond colour determination

Table 2.3.1 shows that for the four avocado fruit samples, the intensity of the yellow colour of avocado oil for the four fractions did not change significantly.

Table 2.3.1: Lovibond colour analyses of avocado oil extracted from unripe and ripe avocado fruit with supercritical carbon dioxide (SC-CO_2)

Sample	Fraction	Blue	Red	Yellow
Unripe, freeze-dried	1	0.05 a*	2.05 ab	19.35 a
	2	0.00 a	2.30 ab	23.00 a
	3	0.85 ab	3.55 cd	21.55 a
	4	4.40 c	4.65 de	25.00 a
Unripe, oven-dried	1	0.00 a	1.60 ab	26.85 a
	2	0.05 a	2.00 ab	26.00 a
	3	3.15 bc	4.10 bcde	27.00 a
	4	5.25 cd	5.50 e	26.10 a
Ripe, freeze-dried	1	0.00 a	1.15 a	21.20 a
	2	0.00 a	1.10 a	20.00 a
	3	0.15 a	1.60 ab	25.15 a
	4	4.25 c	3.45 bcd	24.00 a
Ripe, oven-dried	1	1.45 ab	2.25 ab	26.30 a
	2	0.00 a	1.25 a	23.60 a
	3	1.20 ab	2.60 abc	26.95 a
	4	7.35 d	7.40 f	28.45 a
Pooled standard deviation		0.41	0.29	3.82

* Mean values in the same column followed by the same letter are not significantly different ($p > 0.05$)

However, the intensity of blue and red increased significantly towards the last fractions with the fourth fraction being the greatest in intensity. Similar results have been reported by Przybylski *et al*., (1998) on SC-CO_2-extracted canola oil. They reported increases in measured values of tristimulus (Yellow, Red, Blue) with increasing oil fraction number (i.e. increasing progress of extraction). They attributed this to increases in the amounts of pigments extracted and related it to the chlorophyll and carotenoid content. In this study, the increase in colour intensity of the fourth fractions compared to the earlier ones may also be related to their content of pigments such as chlorophyll and carotenoids.

2.3.3.2 Chlorophyll content

Chlorophyll levels found in the first two fractions in this study (23-58 mg/kg) (Figure 2.3.2) seem to be comparable to levels reported for crude avocado oil (40-60 mg/kg) by Eyres *et al*., (2001). By comparison, refined avocado oil (variety not specified) is reported to have chlorophyll content of 0.3 mg/kg (Smith & Winter, 1970).

The total chlorophyll content of oil from ripe fruit (freeze-dried and oven-dried combined) (1954.57 mg/kg) was lower than that of oil from unripe fruit (2325.94 mg/kg). Ashton *et al*. (2006) also reported a decrease (214 to 116 µg/g oil) in total chlorophyll content of oil extracted from freeze-dried *Hass* avocado peel during ripening (14 days at 20℃). Oxidative enzymes increase during fruit ripening (Prusky, Kobiler, Jacoby, Sims & Midland, 1985; Karni, Prusky, Kobiler, Bar-Shira & Kobiler, 1989), which could lead to oxidative breakdown of chlorophylls, consequently resulting in lower chlorophyll levels in ripe fruit.

There was an increase in the levels of chlorophyll in the oil fractions with progressive extraction. For all the avocado fruit samples, the fourth oil fractions had the highest chlorophyll contents (249.13 – 394.42 mg/kg) followed by the third fractions (78.04 – 210.03 mg/kg) (Figure 2.3.2). The third and fourth oil fractions had higher levels of chlorophyll than the first two fractions, which did not seem to vary significantly in chlorophyll content.

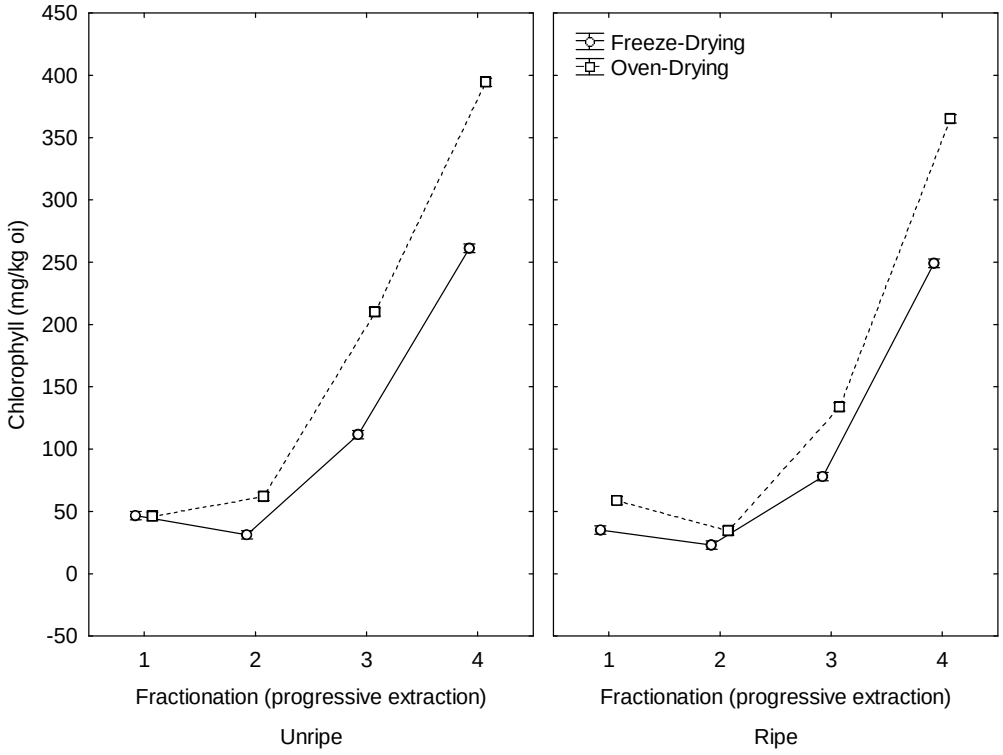


Figure 2.3.2: Chlorophyll content of oil (mg pheophytin/kg oil) extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

In plant cells, chlorophyll is situated in chloroplasts which consist of folded layers of thylakoids (Mauseth, 1995). Parenchyma cells, where the oil is located, only have thin primary walls (Platt & Thomson, 1992). The application of SC-CO_2 in this study was based on pressure forcing the oil out of the plant material. During the early stages of extraction, the first cells to rupture would presumably be the parenchyma cells, thus exposing their oil contents for extraction. After most of the oil has been extracted from these structures, the material may be most likely compacted and the cells with the stronger cell walls, namely the chloroplasts and the idioblasts are then ruptured (Mauseth, 1995), releasing the chlorophyll and terpenoids, respectively (Platt-Aloia *et al*., 1983) in the later oil fractions. This is also demonstrated by the observed increase in intensity of blue and red in the later oil fractions (Table 2.3.1).

With the exception of the first oil fraction from unripe fruit, the oil from oven-dried plant material had higher chlorophyll contents than oil from freeze-dried plant material (Figure 2.3.2).

It has been suggested that two types of reactions are involved in chlorophyll catabolism (Shioi, Tatsumi, & Shimokawa, 1991). Type I reactions involve the loss of phytol (by the action of chlorophyllase) (Tsuchiya, Ohta, Okawa, Iwamatsu, Shimada, Masuda, & Takamiya, 1999), Mg^{2+} (by the action of Mg-dechelatase) (Langmeier, Ginsburg & Matile, 1993; Vicentini, Iten & Matile, 1995), and other modifications of the side chains that do not involve the breaking of the tetrapyrrole ring present in chlorophylls (Huff, 1982). Type II reactions involve the oxidative cleavage (bleaching) of the tetrapyrrole ring with oxidative enzymes such as lipoxygenase (Orthoefer & Dugan, 1973) leading to progressive reduction of the absorbance at 669 nm (Huff, 1982). By production of free radicals and hydroperoxides through its action on polyunsaturated fatty acids, lipoxygenase may oxidize chlorophyll (and other pigments such as carotenoids) (Buckle & Edwards, 1970; Lajolo & Lanfer-Marquez, 1982; Lopez-Ayerra *et al*., 1998). Lipoxygenase is denatured at high temperatures (Harris & Tall, 1994) and could have been inactivated during oven-drying, thus protecting the chlorophyll in oven-dried fruit from being oxidised. On the other hand, during freeze-drying, temperatures did not rise above 25℃ and lipoxygenase would be expected to retain its activity and bring about oxidative bleaching of chlorophyll (Martinez & Labuza, 1968), thus reducing its levels. The resultant effect is higher levels of chlorophyll in oil from oven-dried avocado fruit than from freeze-dried. Furthermore the porosity and surface area of freeze-dried products are greater than those of oven-dried products (Berlin, Kliman & Pallansch, 1996; King, Lam & Sandall, 1968) and might have led to higher oxygen permeability, creating more free radicals (Martinez & Labuza, 1968) resulting in higher oxidative breakdown and lower chlorophyll values for oil extracted from freeze-dried fruit.

The last oil fractions extracted from oven-dried fruit had an olive green colour while those extracted from freeze-dried fruit had a bright green colour (Figure 2.3.3).

Figure 2.3.3: Appearance of oil extracted from ripe avocado fruit with supercritical carbon dioxide (SC-CO_2). A – oil from ripe, oven-dried fruit; B – oil from ripe, freeze-dried fruit.

This was observed for both unripe and ripe fruit. According to Gupte, El_Bisi, and Frances, (1964), conversion of chlorophyll to pheophytins results in a change from bright green to dull olive green or olive yellow. The conversion of chlorophyll to pheophytin due to heat has been well established (Chen & Chen, 1993; Suzuki & Shioi, 2003). Therefore Figure 2.3.3 suggests that the heat treatment during oven-drying may convert chlorophyll pigments to the olive green pheophytins (increasing the absorption at 667-669 nm, the absorption maxima of pheophytin *a* and pyropheophytin *a*) (Von Elbe & Schwartz, 1996). The AOCS method Cc 13i-96 used in this study expresses the content of chlorophyll pigments in terms of pheophytin equivalents (mg pheophytin *a* per kg oil). The visual appearance of the oil from oven-dried and freeze-dried fruit (Figure 2.3.3) therefore agrees with the results in Figure 2.3.2 that show higher levels of chlorophyll pigments (specifically pheophytins) in oil from oven-dried fruit compared to freeze-dried fruit.

Although no significant difference was observed between the yellow values of the treatments (Table 2.3.1), the mean yellow values for oil extracted from oven-dried fruit (calculated to be 26.49 for unripe fruit and 26.33 for ripe fruit) were higher than those of oil extracted from freeze-dried fruit (calculated to be 22.23 for unripe fruit and 22.59 for ripe fruit), possibly indicating the formation of pheophytin as a result of the heat treatment during oven-drying.

2.3.3.3 Carotenoid content

For all oil samples, there was a progressive increase in carotenoid content from the first (6.67 – 15.53 mg/kg) to the last fractions (58.03 – 70.51 mg/kg), with the later fractions becoming enriched in their content of carotenoids (Figure 2.3.4). This is similar to the trend of increasing colour intensity (Table 2.3.1) and increasing levels of chlorophyll (Figure 2.3.2) with increasing oil fractions observed above. A similar enrichment of carotenoid content by progressive SC-CO_2 extraction of palm oil has also been reported by Chuang and Brunner (2006). According to Przybylski *et al*. (1998), the colour of canola oil became more intense during extraction and the absorption related to carotenoids and chlorophyll increased as the extraction proceeded. Carotenoids and chlorophyll are located within the chloroplasts and chromoplasts (Mauseth, 1995), and so they may be extracted simultaneously.

Figure 2.3.4 shows that the carotenoid content of oil extracted from unripe, oven-dried fruit was lower than that of oil extracted from unripe, freeze-dried fruit for the four fractions. For ripe fruit on the other hand, carotenoid content of oil extracted from oven-dried material was higher than from freeze-dried fruit. These observations may be explained by two main factors: the action of lipoxygenase and the destructive effect of heat (during oven-drying) on carotenoids. Unripe avocado fruit have relatively lower levels of lipoxygenase activity (compared to ripe fruit) (Prusky *et al*., 1985; Karni *et al*., 1989).

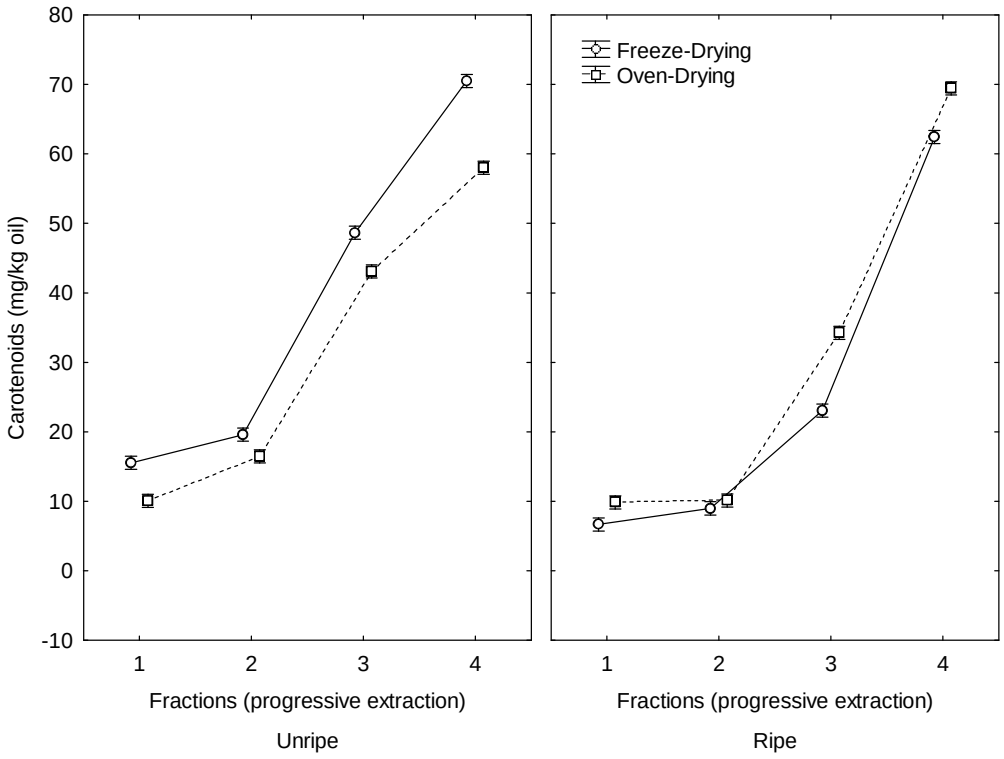


Figure 2.3.4: Carotenoid content (mg/kg oil) of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

The effect of heat may therefore be the important factor in determining the fate of carotenoids in unripe fruit. Oil from unripe, oven-dried fruit would have lower carotenoid content than from unripe, freeze-dried fruit due to destruction of carotenoids by heat during oven-drying.

Fruit ripening has been shown to lead to decreases in carotenoid content in avocado oil. Ashton *et al*. (2006) reported a decrease due to ripening in the total carotenoid content of oil extracted from the peel (200-100 mg/kg) and mesocarp (green flesh: 23-10 mg/kg and yellow flesh: 15-1 mg/kg) of freeze-dried *Hass* avocado. This may be as a result of the action of lipoxygenase due to its relatively higher activity in ripe fruit. Free radicals produced from the action of lipoxygenase on unsaturated fatty acids are able to oxidize carotenoids (Lee & Min, 1990). Application of heat during

oven-drying of the ripe fruit inactivates the lipoxygenase enzyme thus reducing its activity and ability to destroy carotenoids (Lopez-Ayerra *et al*., 1998). Lipoxygenase activity is however, preserved in the ripe, freeze-dried fruit. The resultant effect then is higher carotenoid content of oil from ripe, oven-dried fruit compared to oil from ripe, freeze-dried fruit. The relatively lower levels of carotenoids for all the fractions combined in oil from ripe, freeze-dried fruit (145.83 mg/kg) compared to the other fruit samples (unripe, freeze-dried 308.59 mg/kg; unripe, oven-dried 255.35 mg/kg and ripe, oven-dried 247.31 mg/kg), is a further demonstration of the importance of lipoxygenase enzyme activity in determining the levels of carotenoids in oil from the ripe fruit.

2.3.3.4 Unsaponifiable content

Figure 2.3.5 shows that apart from ripe, oven-dried fruit which had similar unsaponifiable content in all four oil fractions, the rest of the fruit samples had higher unsaponifiable content in oil Fraction 1 than their later fractions. This is in contrast with the chlorophyll (Figure 2.3.2) and carotenoid (Figure 2.3.4) contents that increased with progressive extraction. When the carotenoid, chlorophyll, tocopherol and sterol values obtained during analyses were added together, the average total value was 0.40 g/100 g for freeze-dried fruit (Figure 2.3.6a) and 0.45 g/100 g for oven-dried fruit (Figure 2.3.6b). The values were notably lower than those obtained with the analytical method for determination of unsaponifiables (average of freeze-dried fruit: 2.79 mg/ 100g oil; average of oven-dried fruit: 2.92 mg/ 100g oil). This is an indication that there may have been a component of the unsaponifiable fraction that was not assayed.

Although the chlorophyll, carotenoids, sterols and tocopherols form part of the unsaponifiable fraction, there are other non-polar hydrocarbon-like substances like waxes, which could have eluted mainly during the early stages of extraction with SC-CO_2, and may account at least in part for the part of the unsaponifiable fraction not analysed. Waxes are non-polar (Gunstone, 1996), occur on the surface of the fruit skin (Mauseth, 1995) and would be highly soluble (Gunstone, 1996) in the non-polar SC-CO_2 (Jikei, Saitoh, Yasuda, Itoh, Sone, Kakimoto, & Yoshida, 2006).

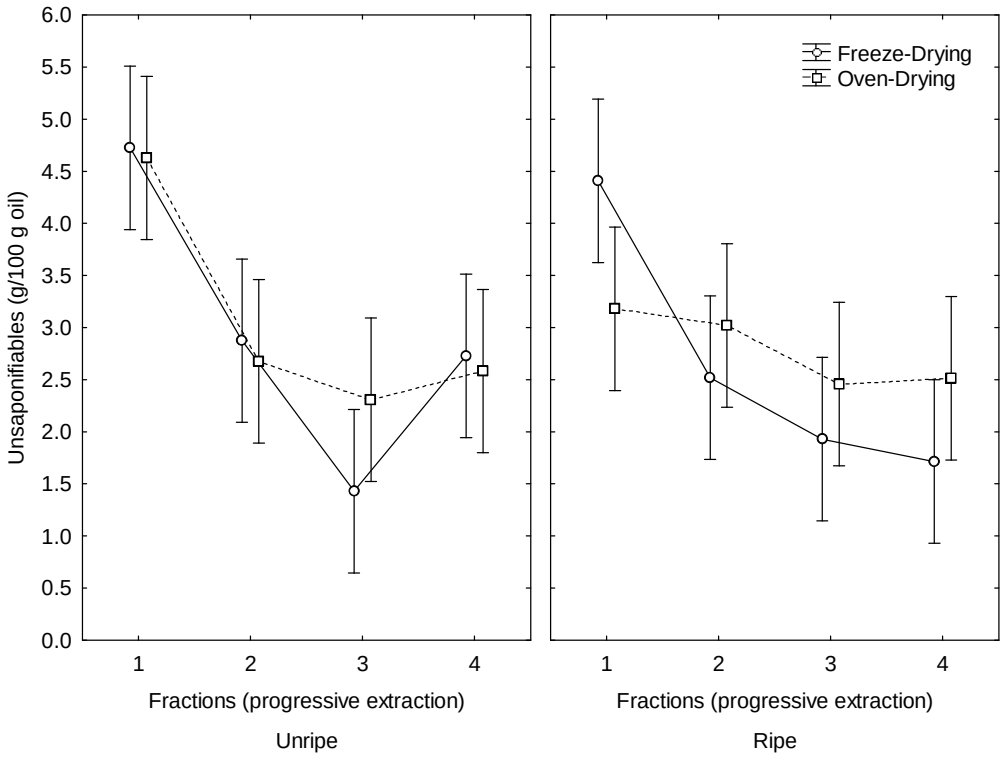


Figure 2.3.5: Unsaponifiable content (g/100 g oil) of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

It is therefore likely, that the non-polar waxes would have been extracted early due to their availability to the solvent as well as their similar polarity to the solvent.

It was expected that the unsaponifiable content of oil from oven-dried avocado, would be higher than that of freeze-dried avocado. Farines *et al*. (1995) reported the formation of a class of compounds with a furyl nucleus attached to a long aliphatic chain during heat drying of avocados, which tend to increase the unsaponifiable content by up to 50%. These compounds, if present, could have led to an increase in unsaponifiable content in oil from oven-dried fruit. However, no increase in unsaponifiable matter in oil extracted from oven-dried fruit was observed in this study.

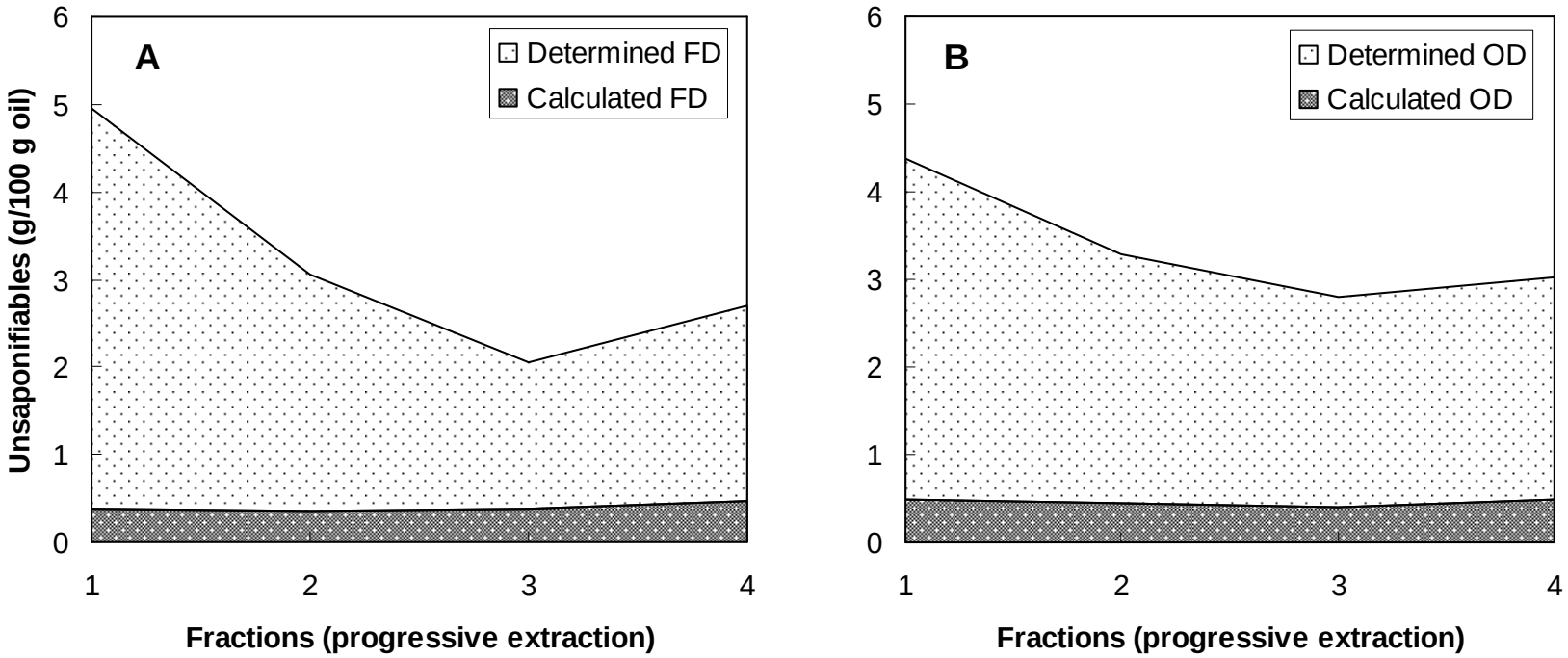


Figure 2.3.6: Unsaponifiable values determined with AOCS method Ca 6a-40 and unsaponifiable values calculated by adding the micro-components (carotenoids, chlorophyll, tocopherols and sterols) together for oil extracted from freeze-dried (a) and oven-dried (b) avocado fruit with supercritical carbon dioxide (SC-CO_2). FD (Freeze dried), OD (Oven dried).

2.3.3.5 Tocopherols

For all avocado oil samples, the levels of individual tocopherol isomers varied over a wide range (Figure 2.3.7). In all oil fractions from all fruit samples, the amount of β-tocopherol was the lowest of the four tocopherol isomers. Oil from ripe, freeze-dried fruit had relatively lower levels of tocopherols, in particular, α-, γ- and δ. Levels of total tocopherols in oil from ripe, freeze-dried fruit were lower (a range of 11.78 – 21.39 mg/100g) compared to oil from the other fruit samples (a range of 28.94 – 40.58 mg/100g).

Higher values were obtained by Lozano *et al*., (1993), who reported a total tocopherol content of 57.0 – 103.0 mg/100g oil for crude avocado oil extracted from freeze-dried, unripe mesocarop (*Fuerte* being the lowest) (Lozano *et al.*, 1993). However, lower levels (13.0 - 20.0 mg/100g oil) have been reported for centrifugal extracted avocado oil from an unknown variety (Eyres *et al.,* 2001).

The low total tocopherol levels in oil from ripe, freeze-dried fruit may be due to the relatively higher lipoxygenase activity of the ripe, freeze-dried fruit. Free radicals created by lipoxygenase will not only attack carotenoids, but also tocopherol, which is also a free radical scavenger (Palozza & Krinsky, 1992).

Przyblylski *et al*. (1998), found α- and δ-tocopherol in almost equal proportions in the last three fractions of SC-CO_2-extracted canola oil, with α-tocopherol being higher and δ-tocopherol being lower in the first fractions. In this study, no specific trend was observed for either of these isomers in the different fractions.

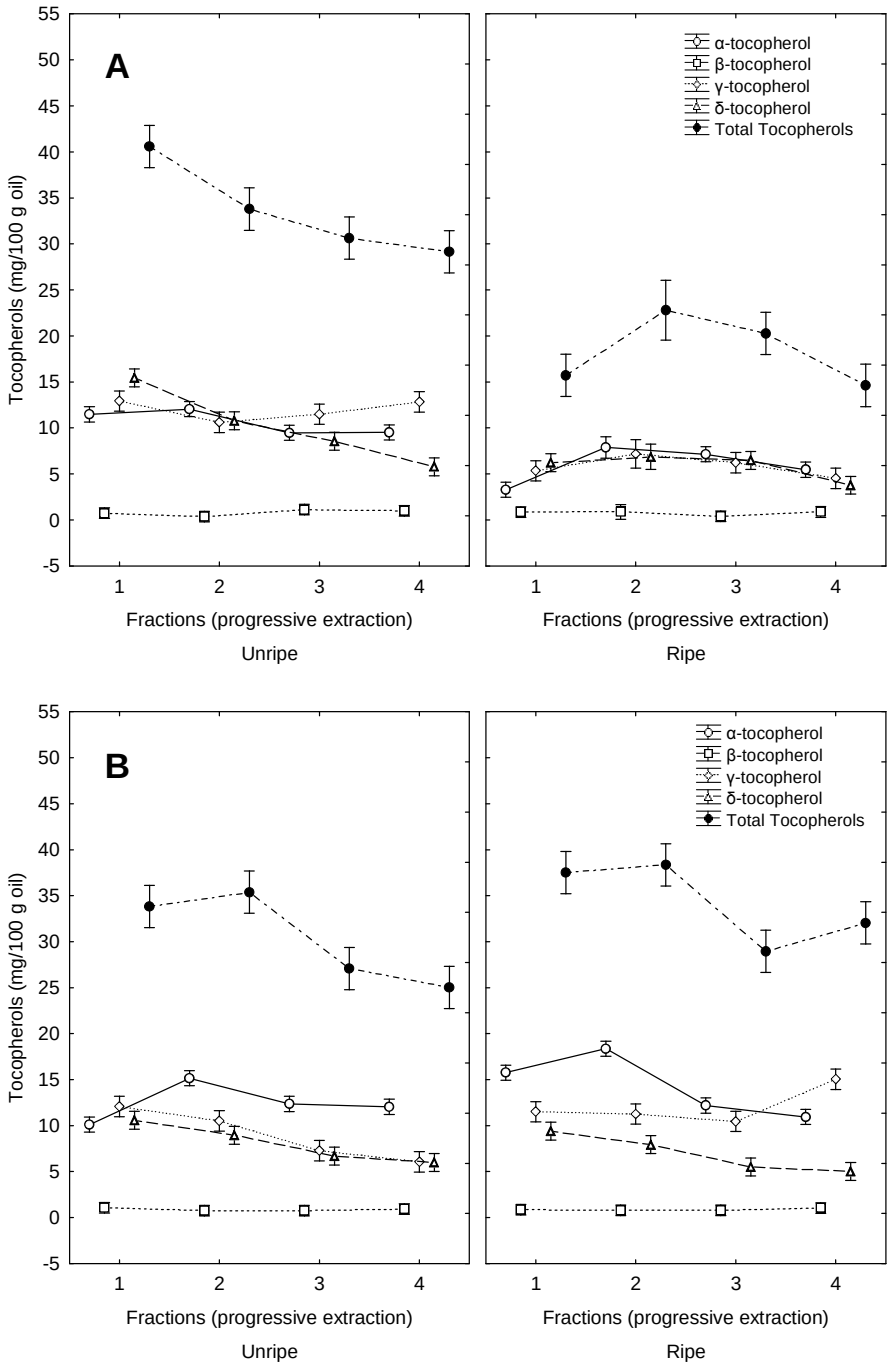


Figure 2.3.7: Tocopherol content of oil extracted from (A) freeze-dried and (B) oven-dried avocado fruit with supercritical carbon dioxide (SC-CO_2). (Total = alpha + beta + gamma + delta). Vertical bars denote 0.95 confidence intervals.

2.3.3.6 Sterol content

In all samples, β-sitosterol (range of 2710.9 – 3716.3 mg/kg oil) occurred in the greatest proportion relative to total sterols (range of 3277.5 – 4373.2 mg/kg oil), followed by δ-5-avenasterol (range of 227.7 – 441.7 mg/kg oil) and campesterol (range of 154.1 – 259.3 mg/kg oil) (Table 2.3.2). Most vegetable oils contain 0.1 to 0.5 % sterols (1000 – 5000 mg/kg) (Shahidi & Senanayake, 2006). The levels of total sterols (3277.5 – 4373.2 mg/kg oil) falls within this range. Lozano *et al*. (1993) reported a total sterol content of 0.9% in hexane-extracted oil from freeze-dried *Fuerte*. Beta-sitosterol is generally the major phytosterol, contributing 50 to 80 % to the total content of sterols, with campesterol and stigmasterol usually present in significant levels (Shahidi & Senanayake, 2006). The other sterols occurred in low amounts or were not detectable. Specifically, δ-7-avenasterol was not detectable in oil from unripe, freeze-dried fruit, the first three oil fractions of unripe, oven-dried fruit and first two fractions of oil from ripe, oven-dried fruit.

Delta-7-stigmasterol was not detectable in the third oil fraction from ripe, freeze-dried fruit and the first two oil fractions of ripe, oven-dried fruit. Stigmasterol was not detected in oil from unripe, freeze-dried fruit and the first three oil fractions of unripe, oven-dried fruit. Campestanol was not detected in oil from unripe fruit (freeze-dried and oven-dried) and ripe, freeze-dried fruit and could have been formed from campesterol in oil from ripe, oven-dried fruit. The conversion of campesterol to campestanol involves the action of 5α-reductase in the C-6 oxidation pathway in plants and has been studied in Arabidopsis (Fujioka, Li, Choi, Seto, Takatsuto, Noguchi, Watanabe, Kuriyama, Yokota, Chory, & Sakurai, 1997; Crozier *et al*., 2000). Sánchez, Osoria Montaño & Martinez, (2004) observed an increase in campesterol during ripening of olives. This was not observed in the current study. If the values obtained for campestanol in oil from ripe oven-dried fruit were to be added to the values obtained for campesterol values for the same samples and regarded as total campesterol, relatively higher campesterol values would be observed for campesterol in oil from oven-dried unripe (215.18mg/ kg oil) and ripe fruit (258.21mg/ kg oil) than for freeze-dried unripe (178.20mg/ kg oil) and ripe fruit (185.43mg/ kg oil). This may indicate that these sterols were protected by

Table 2.3.2: Sterol content (mg/kg oil) of avocado oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2)

Sample	Fraction	δ-7-avenasterol	δ-7-stigmasterol	δ-5-avenasterol	β-sitosterol	Stigmasterol	Campestanol	Campesterol	Total sterols
UFD	1	ND*	41.1 a¶	227.7 a	3341.9 a	ND	ND	180.6 a	3791.3 a
	2	ND	47.3 a	228.5 a	2988.0 a	ND	ND	168.1 a	3432.1 a
	3	ND	80.1 a	304.0 a	2822.9 a	ND	ND	169.0 a	3376.0 a
	4	ND	95.7 a	375.9 a	3415.0 a	ND	ND	195.0 a	4081.7 a
UOD	1	ND	83.7 a	358.3 a	3672.0 a	ND	ND	259.3 a	4373.2 a
	2	ND	76.4 a	326.6 a	3408.7 a	ND	ND	208.2 a	4019.9 a
	3	ND	87.5 a	290.7 a	3073.3 a	ND	ND	179.0 a	3630.5 a
	4	8.0 a	97.9 a	381.2 a	3467.7 a	18.3 abc	ND	214.2 a	4198.8 a
RFD	1	41.7 a	13.2 a	293.7 a	2709.7 a	36.4 bc	ND	182.7 a	3277.5 a
	2	43.7 a	22.3 a	266.4 a	2750.6 a	40.1 c	ND	195.0 a	3318.1 a
	3	40.3 a	ND	312.0 a	2762.5 a	25.7 abc	ND	163.7 a	3304.2 a
	4	6.1 a	41.7 a	441.7 a	3557.6 a	4.7 ab	ND	200.3 a	4252.2 a
ROD	1	ND	ND	349.9 a	3716.3 a	22.1 abc	93.7 b	171.8 a	4353.9 a
	2	ND	ND	293.4 a	3318.8 a	16.3 abc	107.2 b	176.7 a	3912.4 a
	3	7.5 a	90.9 a	295.6 a	2925.0 a	6.5 abc	82.7 ab	154.1 a	3562.2 a
	4	8.2 a	93.0 a	361.3 a	3289.6 a	5.2 abc	77.6 ab	169.1 a	4004.0 a
Pooled standard deviation		7.8	17.4	42.4	403.5	6.0	14.7	23.0	467.8

* ND – Not detected

¶Mean values in the same column followed by the same letter are not significantly different ($p > 0.05$)

UFD – unripe, freeze-dried; UOD – unripe, oven-dried; RFD – ripe, freeze-dried; ROD – ripe, oven-dried

compounds with antioxidant activity possibly formed during oven drying. Ripening of climacteric fruit is associated with an increase in the levels of various enzymes (Prasanna, Prabha & Tharanathan, 2007). This would mean that if the campestanol was formed from campesterol due to increased levels of 5α-reductase in ripe fruit, the conversion should have been visible in freeze-dried fruit too. Therefore the observed presence of campestanol only in oil from ripe, oven-dried fruit suggests that temperature may be an important factor. Some reductase enzymes have been reported to have optimum activities at temperatures of 25 – 60 ℃ in various crops (Chopra, 1983; Wendroth & Seitz, 1990). It could be hypothesised therefore, that the enzymatic conversion of campesterol to campestanol may have been initially induced by the higher temperatures when the fruit pieces were introduced into the warm oven, resulting in the production of campestanol. This could possibly explain why no campestanol was detected in oil from ripe, freeze-dried fruit, probably because the prevailing temperature conditions during freeze-drying were too low to induce this reaction

Frega *et al*., (1993) detected campesterol (280 mg/kg), stigmasterol (20 mg/kg), β-sitosterol (8140 mg/kg) and δ-5-avenasterol (900 mg/kg) in avocado oil from an unknown origin. In this study, total sterol levels were similar in all four oil fractions from the four fruit samples.

2.3.3.7 Oxidative stability index (OSI)

The results obtained for the OSI have already been shown (Figure 2.2.8, p. 69) and discussed. Here, the possible effects of micro-components on the OSI will be discussed.

The induction period for the first two oil fractions of ripe, freeze-dried fruit (1.08 – 3.01 h) was lower while the values for ripe, oven-dried fruit were slightly higher (3.04 – 9.17 h) (Figure 2.2.8, p. 69). Relatively lower temperatures used during freeze-drying may preserve the activity of enzymes such as lipoxygenase which could destroy antioxidants (Park, 1987) like carotenoids and tocopherols and could have decreased the oxidative stability of the oil (Boscovic, 1979) from freeze-dried fruit.

Elevated temperatures during oven-drying may inactivate lipoxygenase enzymes and could have helped to stabilize the oil. Higher temperatures could also have inactivated lipases which lead to hydrolytic degradation by releasing free fatty acids (Harris & Tall, 1994).

2.3.3.8 Correlations

High, positive correlations were found between chlorophyll and blue (0.96), chlorophyll and red (0.92), carotenoids and blue (0.82) and carotenoids and red (0.85) (Table 2.3.3). These results suggest that chlorophyll and carotenoids contribute significantly to the colour of the oil samples. The colorimetric index is often used to monitor the occurrence or change of pigments acting as pro- or antioxidants (Low, Lee & Kong, 1998). There was a high positive correlation (0.83) between chlorophyll and the OSI (Table 2.3.3), which indicates that the chlorophyll exerted an antioxidant effect in the oils. Although chlorophyll is a well-known pro-oxidant, it seems to have antioxidant potential when the autoxidation reaction occurs in the dark (Endo *et al*., 1985[a]; Endo *et al*., 1985[b]; Zambiazi, & Przybylski, 1998; Psomiadou & Tsimidou, 2002).

Psomiadou and Tsimidou (2002) observed the formation of a derivative of pheophytin *a* (pyropheophytin) after oven-treatment at 40 and 60℃ in olive oil. Pyropheophytin is reported to have a strong antioxidant activity (Cahyana Shuto & Kinoshita, 1992), and could have contributed to increased induction periods on the Rancimat after addition of pheophytin to the olive oil (Psomiadou & Tsimidou, 2002). Pheophytin and pyropheophytin might have had the same effect in this study.

A high positive correlation (0.80) was found between carotenoid content and the OSI (Table 2.3.3). This may be attributed to the antioxidant power of carotenoids. Carotenoids are well-known antioxidants in oil systems, especially in combination with tocopherol (Farombi & Britton, 1999; Henry, Catignani & Schwartz, 1998; Palozza & Krinsky, 1992). It is important to conduct further analyses to determine whether carotenoids function synergistically with other compounds in the oil in exerting antioxidant effects.

Table 2.3.3: Correlation coefficients between micro-components, colour and oxidative stability index (OSI) of avocado oil extracted with supercritical carbon dioxide (SC-CO_2)

	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	Total tocopherols	chlorophyll	carotenoids	unsapoifialbes	campesterol	campestanol	stigmasterol	β-sitosterol	δ-5-avenasterol	δ-7-stigmasterol	δ-7-avenasterol	Total sterols	blue	red	yellow	OSI
α-tocopherol	1.00																			
β-tocopherol	-0.06	1.00																		
γ-tocopherol	0.50**	0.15	1.00																	
δ-tocopherol	0.34	-0.13	0.45*	1.00																
Total tocopherols	0.82***	0.03	0.83***	0.71***	1.00															
chlorophyll	-0.11	0.23	-0.05	-0.59***	-0.28	1.00														
carotenoids	-0.10	0.20	0.18	-0.47***	-0.14	0.84***	1.00													
unsaponifiables	0.01	0.03	0.22	0.53**	0.29	-0.39*	-0.45**	1.00												
campesterol	-0.04	0.31	-0.03	0.12	0.02	0.08	-0.05	0.34	1.00											
campestanol	0.57***	0.08	0.41*	-0.11	0.41*	-0.01	-0.06	-0.02	-0.31	1.00										
stigmasterol	-0.25	-0.13	-0.45*	-0.23	-0.40*	-0.19	-0.31	0.10	-0.14	0.14	1.00									
β-sitosterol	0.31	0.28	0.20	0.13	0.30	0.20	0.04	0.15	0.68***	0.19	-0.24	1.00								
δ-5-avenasterol	-0.19	0.37*	-0.21	-0.54**	-0.36*	0.55**	0.35**	-0.30	0.48**	0.02	0.00	0.60***	1.00							
δ-7-stigmasterol	0.04	0.28	0.27	-0.16	0.09	0.60***	0.67***	-0.14	0.39*	-0.20	-0.62***	0.28	0.29	1.00						
δ-7-avenasterol	-0.58***	-0.20	-0.51**	-0.29	-0.61***	-0.17	-0.19	0.01	-0.19	-0.18	0.82***	-0.47**	-0.08	-0.46**	1.00					
Total sterols	0.27	0.32	0.17	0.02	0.22	0.28	0.11	0.10	0.70***	0.21	-0.20	0.99***	0.69***	0.33	-0.42*	1.00				
blue	-0.09	0.24	0.06	-0.57***	-0.22	0.96***	0.82***	-0.33	0.04	0.10	-0.16	0.25	0.58***	0.52**	-0.16	0.33	1.00			
red	0.00	0.25	0.22	-0.42*	-0.06	0.92***	0.85***	-0.34	-0.02	0.06	-0.32	0.16	0.41*	0.64***	-0.28	0.22	0.94***	1.00		
yellow	0.37***	-0.05	0.32	-0.06	0.28	0.14**	0.35	0.01	0.02	0.26	0.01	0.00	-0.07	0.30	-0.04	0.04	0.15	0.18	1.00	
OSI	0.17	0.13	0.10	-0.60***	-0.09	0.83***	0.80***	-0.53***	-0.18	0.26	-0.26	0.00	0.28	0.62***	-0.23	0.08	0.78***	0.83***	0.32**	1.00

*,**,*** indicate significance at $p < 0.05$, 0.01 and 0.001, respectively.

The correlation between total tocopherols and OSI was not significant (Table 2.3.3). The only significant correlation between the OSI and any of the tocopherol isomers, including total tocopherol, was for δ-tocopherol (–0.60), suggesting that this isomer exerted a pro-oxidant effect. However, this may be due to a slight decrease in the extraction profile of the δ-tocopherol relative to the other isomers, and could be a chance correlation rather than an indication of pro-oxidant action of the isomer.

Correlations between OSI and individual sterol isomers were not significant except in the case of δ-7-stigmasterol where there was a significant positive correlation (0.62) (Table 2.3.3). Correlation between total sterols and OSI was not significant. Sterols such as fucosterol, δ-5-avenasterol, vernosterol, fucosterol and citrostadienol have been shown to have antioxidant activity in oils at 180ºC (Rajalakshmi & Narasimhan, 1995; White & Armstrong, 1986) whereas stigmasterol and cholesterol did not exhibit any antioxidant activity (Gordon & Magos, 1983). The positive correlation between δ-7-stigmasterol and oxidative stability index may indicate that this particular sterol may be exerting some antioxidant effects. Although several studies have confirmed the stabilizing effect of vernosterol, δ-7-avenasterol, δ-5-avenasterol in oil subjected to prolonged heating (Sims *et al*., 1972; Boskou & Morton, 1976; White & Armstrong, 1986), it was not observed in this study.

A significant negative correlation (–0.53) was found between the unsaponifiable matter and the OSI (Table 2.3.3). This may suggest the presence of a pro-oxidant in the unsaponifiable matter of the first fractions of the oil that may be decreasing with progressive extraction, thus enhancing the oxidative stability of the oil. It may also be a chance correlation due to the suspected high levels of waxy material in the first fractions. However, it is important to note that the unsaponifiable fraction consists of a wide range of compounds, some exhibiting pro-oxidant or antioxidant effects. The contribution of the unsaponifiable fraction to oxidative stability of the oil is a resultant of these pro-oxidant and antioxidant effects of the individual compounds, including possible synergistic interactions.

2.3.3.9 Modelling

Using multiple regression techniques, predictive models were developed to determine the effect of the micro-components on the oxidative stability of the oil. The aim was to set up a model to predict the oxidative stability index (OSI) of avocado oil based on compositional micro-component indices. The OSI has been proven to be a good indicator of shelf-life of edible oils (Van der Merwe, 2003).

The models were based on the following equation:

$OSI = B_0$ (intercept) + $B_1 Variable_1 + B_2 Variable_2 + B_i Variable_j$

Where

B_i Regression coefficients

$Variable_j$ Independent variables, consisting of micro-components such as sterols, tocopherols, carotenoids etc. selected for the model by multiple regression.

Model 1

All the data obtained from the analyses of micro-components (α-, β-, γ-, δ- and total tocopherols, campesterol, campestanol stigmasterol, β-sitosterol, δ-5-avenasterol, δ-7-stigmasterol, δ-7-avenasterol, total sterols, carotenoids and chlorophyll content) were used as independent variables to create model 1. Stepwise regression with forward selection was used to obtain the model. An F-to-enter of 2 was used. The statistical parameters obtained from the multiple regression process for the model are shown in Table 2.3.4. The graph of the predicted versus the observed values is shown in Figure 2.3.8A.

Chlorophyll was the first variable to be selected by the model. This means that chlorophyll, on its own, was the best predictor of the OSI when compared to all the other variables on their own. Therefore, the importance of chlorophyll as an antioxidant under the conditions of this study seems to be clear according to this model.

Table 2.3.4: Statistical parameters obtained from regression of oxidative stability index as dependent variable and micro-components as independent variables for the three models

N = 31 cases	β (Beta) regression coefficients	B regression coefficients	Standard error of B coefficients	p-level
Model 1				
Intercept		8.57	2.87	0.007
Chlorophyll	0.36	0.02	0.01	0.006
Campestanol	0.44	0.67	0.08	0.000
δ-7-stigmasterol	0.42	0.68	0.10	0.000
Total sterols	-0.27	-0.03	0.01	0.000
γ- tocopherol	-0.25	-0.50	0.11	0.000
α- tocopherol	0.37	0.61	0.11	0.000
Carotenoids	0.38	0.11	0.03	0.003
δ-7-avenasterol	0.35	1.36	0.37	0.002
Stigmasterol	-0.22	-1.07	0.47	0.033
¶R^{2}¶ = 0.97; F (9,21) = 82.1; Standard error of estimate = 1.27				
Model 2				
Intercept		7.83	4.63	0.010
Chlorophyll	0.97	0.05	0.01	0.000
Total sterols	-0.24	-0.03	0.01	0.029
Total tocopherols	0.23	0.19	0.08	0.031
R^2 = 0.77; F (3,27) = 29.42; Standard error of estimate = 3.25				
Model 3				
Intercept		2.09	1.24	0.010
Carotenoids	0.79	0.22	0.03	0.000
R^2 = 0.63; F (1,29) = 29.42; Standard error of estimate = 3.96				

Based on the ß-values, campestanol (followed by δ-7-stigmasterol and carotenoids) make the largest contribution to the prediction of the standardised OSI value. Although no antioxidant activity has been attributed to campestanol, δ-7-stigmasterol or δ-7-avenasterol, these were selected by the program to influence the OSI positively while total sterols and stigmasterol were selected to influence the OSI negatively. From Table 2.3.3, the only significant correlation between the OSI and any of the sterols was for δ-7-stigmasterol (0.62).

δ-5-Avenasterol, which is the most abundant sterol in avocado oil after β–sitosterol could have exerted antioxidant effects (White & Armstrong, 1986) in synergy with some of the sterols and micro-components to increase the OSI. The only tocopherol isomer that had a significant correlation with the OSI was δ–tocopherol (–0.60) (Table 2.3.3) but it was not selected by the model. Instead, α– and γ– tocopherols were selected.

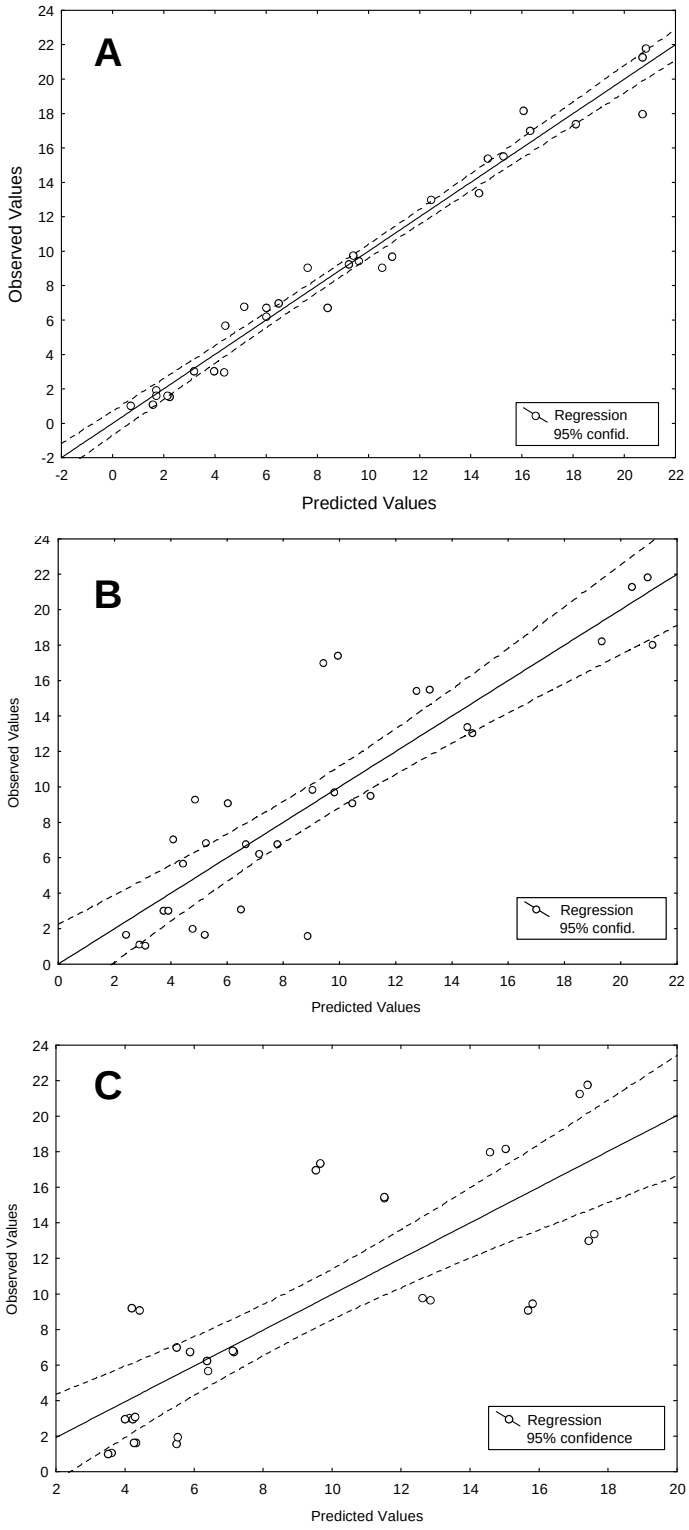


Figure 2.3.8: Predicted versus observed oxidative stability index values based on micro-component content for avocado oil extracted with supercritical carbon dioxide (SC-CO_2) for the three models.

Total sterols, γ-tocopherol and stigmasterol had negative coefficients. This means that, according to the model, they act as pro-oxidants and that their presence decreased the OSI. Especially for γ– tocopherol, this is unusual as γ– tocopherol is considered by some authors as the most effective antioxidant (Gottshein & Grosch, 1990; Pongracz, Weiser & Matzinger, 1995).

These unusual selections indicate that Model 1 may be over-modelled because too many variables were introduced. A good predictive model should ideally not include too many variables as is found in calibration of near infrared instruments (Chung Cho, Toyoda, Nakano, Maeda, 2006). This means that chance relationships will inevitably occur and the correlation coefficient, although very high, will be unrealistic due to these chance relationships. Also, the inclusion of total sterols and total tocopherols as well as the individual sterols and individual tocopherols, could be causing the duplication of information. A solution to this was to reduce the amount of variables as shown in Model 2 below.

Model 2

Model 2 started with only 4 variables namely chlorophyll, carotenoids, total sterols and total tocopherols. The stepwise, multiple regression results are shown in Table 2.3.4. The graph of the predicted versus the observed values is shown in Figure 2.3.8B.

The program again selected chlorophyll first. The β-value (0.77) indicates that chlorophyll had the greatest contribution to the OSI compared to total sterols and total tocopherols. Total sterols had a negative coefficient, which means that it had a pro-oxidant effect according to this model. This suggests that sterols, or one or more compounds with pro-oxidant activity, which were not measured (like metals) correlating by chance with sterols are important factors in the determination of the OSI of avocado oil extracted with SC-CO_2.

The R^2 for Model 2 (0.77) was less than that for Model 1 (0.97). Although the scatter increased and the correlation coefficient decreased, Model 2 could be considered a more realistic model because fewer variables were included and the

model was better associated with the well-established role of tocopherols as antioxidants (Murcia *et al*., 2001

For Model 2, carotenoids were not selected by the program. Because both the chlorophyll (0.83) and carotenoids (0.80) correlated well with the OSI (Table 2.3.3), it was possible that the program had declared the carotenoids redundant because their effects on the OSI were very similar to that of chlorophyll. The redundancy effect was tested in Model 3 by leaving chlorophyll out as a variable.

Model 3

The starting variables included in Model 3 were carotenoids, total sterols and total tocopherols. The exclusion of chlorophyll resulted in only carotenoids being selected by the forward stepwise regression model, thus showing that carotenoids were redundant in Model 2. The multiple regression results are shown in Table 2.3.4. The graph of the predicted versus the observed values is shown in Figure 2.3.8C. Only carotenoids influenced the OSI when chlorophyll was left out.

In summary, the OSI of the avocado oils can be predicted from the levels of chlorophyll (or carotenoids), total sterols and total tocopherols. The models indicate that chlorophyll, the carotenoids and tocopherols (although the tocopherol contribution to the OSI was very low) may act as anti-oxidants and the total sterols as pro-oxidants in the avocado oils extracted with SC-CO_2. The pro-oxidant activity of the sterols may be attributed to the presence of other substances with pro-oxidant effect such as metals which were not analysed in this work but may have had similar elution profiles to the sterols.

2.3.4 Conclusions

The intensity of blue and red, and levels of chlorophyll and carotenoid pigments of avocado oil extracted with SC-CO_2 increase with progressive extraction. This may be related to their location in chloroplasts and idioblast cells which are presumably only ruptured in the latter stages of the extraction process. Levels of tocopherols and sterols do not seem to follow a specific trend with progressive extraction. Oil

from ripe, freeze-dried avocado has low levels of chlorophyll, carotenoids and tocopherols, possibly due to low temperatures used during freeze-drying that do not inactivate lipoxygenase enzyme which is then able to degrade these compounds. The OSI of the oil correlates well with its chlorophyll and carotenoid contents. Predictive mathematical modelling indicate that chlorophyll, carotenoids and tocopherols have a positive influence on the oxidative stability of avocado oil extracted with SC-CO_2, while total sterols seem to have a negative effect.

2.3.5 Acknowledgements

Dr P. J. Van Niekerk is gratefully acknowledged for invaluable assistance with statistics and advice on some scientific and technical aspects.

3. DISCUSSION

The general discussion focuses on the methodology and deliberations concerning the execution of this study. The chapter firstly addresses the extractability of the avocado oil, which was influenced by a variety of factors including the ripeness of the avocado fruit, drying method of the avocado fruit as well as extraction method. Secondly, the factors influencing the micro-component content of the oil are discussed; where after the oxidative stability and the variables influencing it are considered.

3.1 Extractability of Avocado Oil

In the industry, avocado oil is extracted using solvent extraction, mechanical pressing, centrifugation of pulp slurries and enzymatic assisted extraction (Buenrostro & López-Munguia, 1986; Bizimana *et al*., 1993). Hard, unripe fruit are used for solvent and mechanical extraction, while soft, ripe seeded fruit are used for oil separation by centrifugation (Werman & Neeman, 1987). For this study, both unripe and ripe fruit was used and it was decided to cut the fruit into small pieces.

The avocado fruit for this study was cut by two methods namely, manual cutting of ripe fruit and mechanical cutting of unripe fruit. The mechanical cutting simulated the hammer mill which is used in industry to cut the unripe fruit into smaller pieces before oven-drying and extraction by means of cold pressing. Ripe fruit is usually left to become slightly overripe to increase the softness for pulping and consecutive centrifugation. Because the variables had to be kept to a minimum, ripe fruit was carefully cut by hand to resemble the sizes obtained by the mechanical cutter for the unripe fruit. As an increase in particle size is associated with a decrease in extraction yield due to the increase of the mass tranfer resistance between the surface of the seed and the SC-CO_2 (Gómez *et al*., 1996; Bhattacharjee *et al*., 2007), particles were put through a 2 mm sieve to assure a maximum size of 2 mm as well as uniformity throughout the matrix. It is important to remember, that the point of cutting is where enzymatic oxidation in conjunction with lipolysis will commence at a high rate (Belitz *et al*., 2004[a]).

One of the aims of this study was to determine whether the degree of ripeness of the avocado fruit had an effect on extractability of oil with SC-CO_2 as well as hexane. The electron micrographs of avocado fruit showed a loss of cellular integrity for ripe fruit compared to unripe, most probably due to enzymatic breakdown of cellular structure. This could have increased the accessibility of the solvent to the oil in the parenchyma cells. Both hexane and SC-CO_2 extracts of ripe fruit yielded higher oil contents (average yield of 714 and 626 g/kg respectively) than corresponding extracts from unripe fruit (average yield of 653 and 555 g/kg respectively). Figure 3.1 illustrates the degradation of the cell walls by enzymes during ripening, which could lead to increased extractability of the oil (Awad & Lewis, 1980; Reymond & Phaff, 1965; Zauberman & Schiffmann-Nadel, 1972).

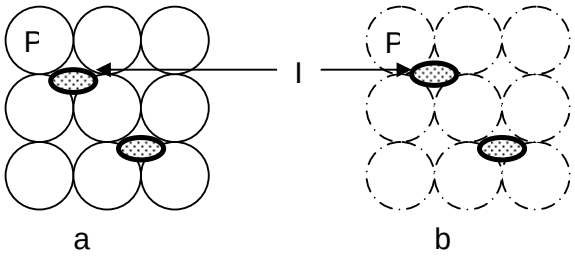


Figure 3.1: Sketch illustrating unripe avocado mesocarp cells where the cell walls of the parenchyma cells (P) are still intact (a), and ripe avocado mesocarp (b) where the parenchyma cell walls have been damaged by cell wall degrading enzymes. The idioblast cells (I) with their thickened cell walls are unaffected by the enzymes.

Water interferes with the effectiveness of solvent extraction of oil and therefore drying is a necessary step prior to extraction (Lewis *et al*., 1978; Sun & Temeli, 2006). Two methods were used for drying the avocado fruit, namely freeze-drying and oven-drying at 80℃. Freeze-drying is a milder technique because it uses lower temperatures and the preservation of compounds and structure is generally associated with this drying technique (Çinar, 2004). Due to the high cost of freeze-drying, the method of drying most commonly used in the oil industry, is oven-drying

or sun-drying. Higher temperatures, however, are known to increase lipid oxidation and this is often enhanced by oven-drying (Galliard, 1994). In this study, oven-dried fruit gave lower oil yields (average of 563 and 668 g/kg) compared to freeze-dried fruit (average of 618 and 699 g/kg) for both SC-CO_2 and hexane extracts respectively.

The avocado fruit used in this study was harvested in August, which represents late season in a South African *Fuerte* season occurring from April to August (Du Plessis, 1980). Horticulturally mature avocado fruit contain a maximum amount during late season of approximately 53 g/kg protein (Vekiari, Papadopoulou, Lionakis, & Krystallis, 2004) and 45 g/kg starch (Liu, Robinson, Madore, Witney, & Arpaia, 1999). All plastids in the avocado mesocarp contain starch and proteins (Platt-Aloia, 1980; Seymour & Tucker, 1993). The high temperatures used during oven-drying of the fruit (80°C) could lead to denaturation of proteins and gelatinisation of starch (Belitz *et al*., 2004[c]). Dehydration could subsequently occur leading to possible crosslinking between proteins, starch and possibly other cell components. The crosslinked structures may then surround oil-containing cellular organelles and reduce their accessibility to the solvent for oil extraction leading to lower oil yields for oven-dried fruit compared to freeze-dried.

As mentioned earlier (Chapter 1, p. 38-39), both oven-dried and freeze-dried fruit samples used in this study had to be pre-frozen mechanically at –20°C before oil extraction. As discussed (Chapter 1, page 47), it is important to note that this may have implications for oil yield. The formation of large ice crystals with sharp edges during the slow freezing process could have damaged the cellular structure of the avocado fruit (Belitz *et al*., 2004[b]). Subsequent oven-drying of this material could have brought about thawing during which the cells would not have retained their structure and the oil might have leaked out of these softened structures during oven-drying. The heat would also have reduced the viscosity of the oil, causing it to leak out of the damaged cells (Fellows, 1990), resulting in higher oil losses during oven-drying. Melting and leaking of oil would have been less likely during the freeze-drying process where relatively lower temperatures and sublimation of the ice crystals were involved.

The advantage of freezing prior to oven-drying was that the pre-treatment prior to the actual drying was the same for both freeze-dried and oven-dried samples and thus, variables were kept to a minimum. Another option would have been the use of quick freezing techniques like cryogenic freezing or high-pressure-shift freezing where structural damage due to the slow formation of large ice crystals can be minimised (Otero, Martino, Zaritzky, Solas, & Sanz, 2000). The formation of smaller ice crystals during cryogenic freezing or high-pressure-shift freezing could lead to less cellular damage (smaller or no holes in the cell membrane), original structure is maintained to a great extent (Otero *et al.*, 2000) and less oil will subsequently leak out during thawing.

In this study, however, a higher oil yield was obtained from freeze-dried samples. Therefore, this might still be the best drying method to use if a high oil yield is the objective and equipment for rapid freezing is not available or too costly. For the industry however, the cost of freeze-drying and oven-drying should be weighed against the oil yield as well as the quality of the oil extracted from this plant material.

The extractability of the avocado oil with SC-CO_2 was compared with hexane (soxhlet extraction) which is the most common solvent used in the industry for extraction of edible oils (Gregory & Horsman, 1997). A laboratory scale SC-CO_2 extractor, which was custom-built, was used to extract the avocado oil on a small scale which was comparable to the quantities used for the soxhlet extraction. The method developed by Botha and McCrindle (2003) was used where only CO_2 was introduced. The small extractor was also more controllable and extractions were more repeatable than the pilot scale extractor used for larger extractions.

The average avocado oil yield obtained from avocado fruit on a dry weight basis was 59.1 % for SC-CO_2 extracts compared to 68.3 % for hexane extracts. The yield obtained from hexane extraction always seems to be more than from SC-CO_2 extraction. Higher oil yields from hexane compared to SC-CO_2 extraction include excess amounts of 1.1 % for borage seed oil (Gómez & de la Ossa, 2002), 0.1 % for soybean oil (Friederich & List, 1982), 0.6 % for grape seed and wheat germ oil (Gómez *et al.*, 1996; Gómez & de la Ossa, 2002), 3.5 %, 2.4 % and 0.8 % for

soybean, sunflower seed and rape seed oil respectively (Stahl, Schutz & Manigold, 1980). The difference in extractability has been explained by the lower selectivity of hexane for pigments and phospholipids (Przybylski *et al*., 1998). This means that hexane will extract more compounds from the oil, resulting in a higher yield, while CO_2 is more selective as a solvent and this will result in smaller oil yield. In this study, the SC-CO_2 extractions were discontinued when an increase in yield of less than 1% per hour was obtained. It is possible that had the SC-CO_2 extractions been extended further, the oil yield could have been similar to the yield from hexane extraction.

The difference in extraction yield between hexane and SC-CO_2 may be explained as follows: Norris (1982) proposed the formation of paths during solvent extraction. According to this author when large extraction chambers are filled with oilseed flakes, there is an inevitable uneven compacting of the material, and consequent channelling of the extraction solvent leading to incomplete oil extraction. Hence solvent extractors are usually fitted with a mechanical mixer to prevent the formation of paths and compacted areas (Norris, 1982). The same concept of channelling of the solvent may be applied to the SC-CO_2 extraction where the formation of paths of least resistance could have led to incomplete extraction (Bhattacharjee *et a*l., 2007). The contents of the SC-CO_2 cylinder are under pressure, with the flow direction of the solvent being the same throughout the extraction and one would expect the formation of a path of least resistance to form readily. Figure 3.2 attempts to illustrate how paths or tunnels of least resistance can be formed by the CO_2 resulting in areas with less CO_2 flow, which end up being compacted or forming “pockets” where some of the oil remain in the plant material. Although this might be true for the soxhlet extraction too, the particles were much less compacted and the method includes boiling steps where the particles are immersed in boiling hexane and sporadically drained, with the solvent constantly flowing in different directions due to the boiling action. It would therefore have been more difficult to form a specific path through the plant material during extraction with hexane.

Figure 3.3 attempts to explain the formation of paths or “tunnelling” on a cellular level, where areas that were compacted might still contain oil whilst cells that fall in the “paths of least resistance” might have been depleted of oil.

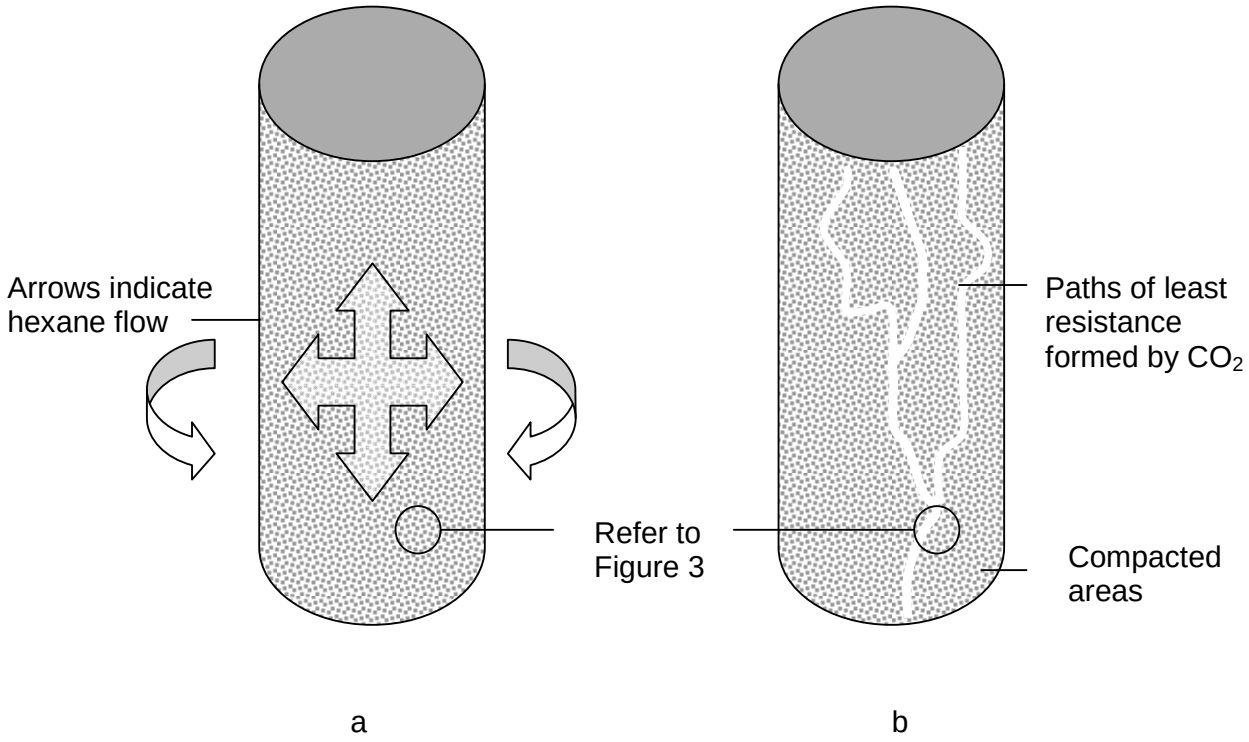


Figure 3.2: Figures illustrating the movement of hexane (a), and supercritical carbon dioxide (SC-CO_2) (b) through the plant material. Hexane flows through and around all particles because the particles are immersed in and boiled in the solvent. SC-CO_2 is forced through with pressure and paths of least resistance may be formed causing areas with less than optimum extraction.

The SC-CO_2 system has the advantages of being environmentally friendly and according to literature it is able to optimize extraction to equal the extraction yield obtained with hexane (Friederich & List, 1982; Gómez & de la Ossa, 2002; Bravi *et al*., 2007). If the process were to be upgraded to a large operational scale, the cost implications will have to be weighed up against the time spent per extraction and ultimately the quality of the oil, which will be addressed later.

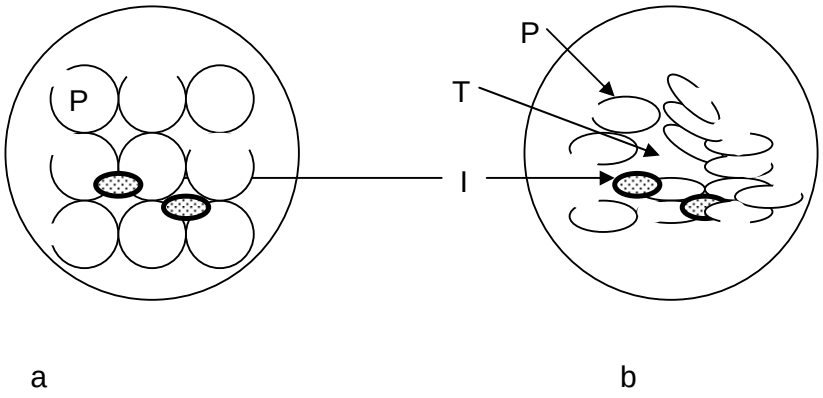


Figure 3.3: Sketch illustrating avocado mesocarp cells after extraction with hexane (a) and after extraction with SC-CO_2 (b) where the cells have been compacted due to the pressure in the cylinder and "tunnels" have been formed where the SC-CO_2 followed the path of least resistance. P = parenchyma cells, I = idioblast cells, T = tunneling effect.

3.2 Effect of fruit ripeness, method of drying and progressive extraction on micro-components of avocado oil

One of the main aims of this study was to characterise the micro-components of the avocado oil extracted with SC-CO_2. For this, a larger, in-house built apparatus was used for oil extraction with SC-CO_2. The extraction was conducted at 350 atm and 45℃. The flow rate was measured as 60 L/h after s eparator 1. In extracting canola oil using SC-CO_2, Przybylski *et al*. (1998), for example, used canola flour with smaller particle size (0.2 - 0.4 mm) than the avocado pieces used in this study (2 mm). Their extraction was carried out at 313 K (39.85 ℃) and 41.4 MPa (408.59 atm) and a flow rate of 10L/ min (600L/h). The pressure and flow rate they used was thus higher, while the temperature was similar to that of the extraction performed in this study. No mention is made of how the canola seeds were dried in this study. It is therefore difficult to make direct comparisons between the results obtained by Przybylski *et al*. (1998) and results of the present study of how drying method influenced micro-components of the oil extracted with SC-CO_2. Because the canola seed was harvested at one maturity level (Przybylski *et al*., 1998), the effect of ripeness on micro-component levels in the avocado oil extracted in this

study could also not be compared to that of the canola oil. However, some authors have reported that some micro-components decrease during ripening in avocado fruit (Ashton *et al*., 2006).

The previous paragraph is an illustration of the difficulty in comparing extractions where different temperatures, pressures and other parameters are used. Furthermore, the particle size, oil content, type of product, micro-component content of the raw product and pre-treatment also play important roles in the micro-component content of the extracted oil. The effect of progressive extraction on the micro-component levels in oil could, however, be compared to an extent with effects observed in other studies and will be discussed later in this section.

Drying method and ripeness of the fruit influenced levels of all tocopherol homologues and total tocopherols significantly, with the exception of β-tocopherol (Table 3.1). Delta-tocopherol was also not influenced by drying method. Even though the statistics showed significant effects of drying method and fruit ripeness, the levels of the α-, γ- and δ-tocopherol homologues were essentially within the same range especially for oil from unripe, freeze-dried fruit and from all oven-dried fruit samples (Figure 2.3.7, p. 100).

On the whole, there were two significant observations from the tocopherol results. Firstly, the levels of β-tocopherol were always the lowest in all treatments and in all oil fractions. This isomer is usually present in the lowest levels of all the tocopherol homologues in plant oils, except in wheat germ oil, where only α-tocopherol is present in higher levels (Papas, 2006).

Table 3.1: Effect of the variables (ripeness, drying and progressive extraction) on the micro-components and oxidative stability of avocado oil extracted with Supercritical carbon dioxide (SC-CO_2). (p values obtained with ANOVA test of significance)

	Ripeness	Drying	Progressive extraction	Ripeness* Drying	Ripeness* Progressive extraction	Drying* Progressive extraction	Ripeness* Drying* Progressive extraction
α- Tocopherol	0.000	0.000	0.000	0.000	0.016	0.000	0.000
β- Tocopherol	0.845	0.498	0.578	0.872	0.539	0.974	0.404
γ- Tocopherol	0.000	0.000	0.005	0.000	0.000	0.015	0.000
δ- Tocopherol	0.000	0.058	0.000	0.000	0.000	0.029	0.000
Total Tocopherols	0.000	0.000	0.000	0.000	0.001	0.009	0.000
Chlorophyll	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Carotenoids	0.000	0.036	0.000	0.000	0.000	0.000	0.000
Unsaponifiables	0.155	0.495	0.000	0.912	0.137	0.104	0.208
Campesterol	0.026	0.249	0.057	0.004	0.390	0.354	0.522
Campestanol	0.000	0.000	0.755	0.000	0.755	0.755	0.755
Stigmasterol	0.000	0.036	0.039	0.000	0.000	0.010	0.882
β- Sitosterol	0.325	0.042	0.065	0.721	0.918	0.273	0.649
δ- 5 Avenasterol	0.328	0.104	0.000	0.069	0.908	0.021	0.762
δ- 7 Stigmasterol	0.000	0.002	0.000	0.611	0.444	0.094	0.002
δ- 7 Avenasterol	0.000	0.000	0.404	0.000	0.062	0.011	0.101
Total Sterols	0.497	0.027	0.053	0.918	0.954	0.281	0.836
Blue	0.583	0.000	0.000	0.051	0.000	0.001	0.003
Red	0.000	0.000	0.000	0.000	0.000	0.000	0.001
Yellow	0.855	0.000	0.006	0.634	0.051	0.138	0.139
PV	0.000	0.000	0.000	0.000	0.000	0.000	0.000
AV	0.000	0.000	0.000	0.000	0.000	0.000	0.000
TOTOX	0.000	0.000	0.000	0.000	0.000	0.000	0.000
FFA	0.429	0.000	0.000	0.000	0.082	0.000	0.000
OSI	0.011	0.000	0.000	0.000	0.002	0.000	0.000

A second significant observation was that levels of total tocopherols in oil from ripe, freeze-dried fruit were the lowest of all the oil samples representing the different pre-treatments (Figure 2.3.7, p. 100). Also, for ripe fruit, oil from freeze-dried samples had lower total tocopherol levels compared to oil from oven-dried samples. This difference was not observed for unripe fruit. Tocopherols can be affected by free radicals (specifically, peroxy radicals), created by the action of lipoxygenase enzymes on linoleic and linolenic acid (which are preferred substrates). These radicals, when present can attack and break down tocopherols.

There is a discrepancy in literature concerning the antioxidant effect of the different homologues. Hoffman (1989) claims that δ-tocopherol is the most potent antioxidant of the homologues. It is however, generally assumed that α-tocopherol has lower antioxidant activity than γ-tocopherol (Belitz *et al*., 2004[a]). Lampi, Kataja, Kamal-Eldin and Vieno (1999), found that α-tocopherol was a more effective antioxidant at low concentrations (≤ 50 ug/g oil) while γ-tocopherol was the more effective antioxidant at higher concentrations (> 100 ug/g oil) in rapeseed oil. In the current study levels of α-tocopherol were higher than γ-tocopherol in oil from oven dried fruit whilst they were similar in oil from freeze-dried fruit. This suggests that α-tocopherol may react at a slower rate under the conditions of heating applied in this study and therefore γ-tocopherol may be a more effective antioxidant under these conditions.

During the reaction of peroxy radicals with α-tocopherol the chroman ring system opens and an alkyl radical is formed, which in turn oxidizes to a hydroxyalkylquinone (Figure 3.4) (Belitz *et al*., 2004[a]). The radical can start autoxidation of unsaturated fatty acids and therefore higher concentrations of α-tocopherol can have a prooxidant effect. During the autoxidation of γ-tocopherol peroxy radicals abstract a hydrogen from tocopherol causing the formation of a chromanoxyl radical, that can transform into a chromanyl radical (Figure 3.5).

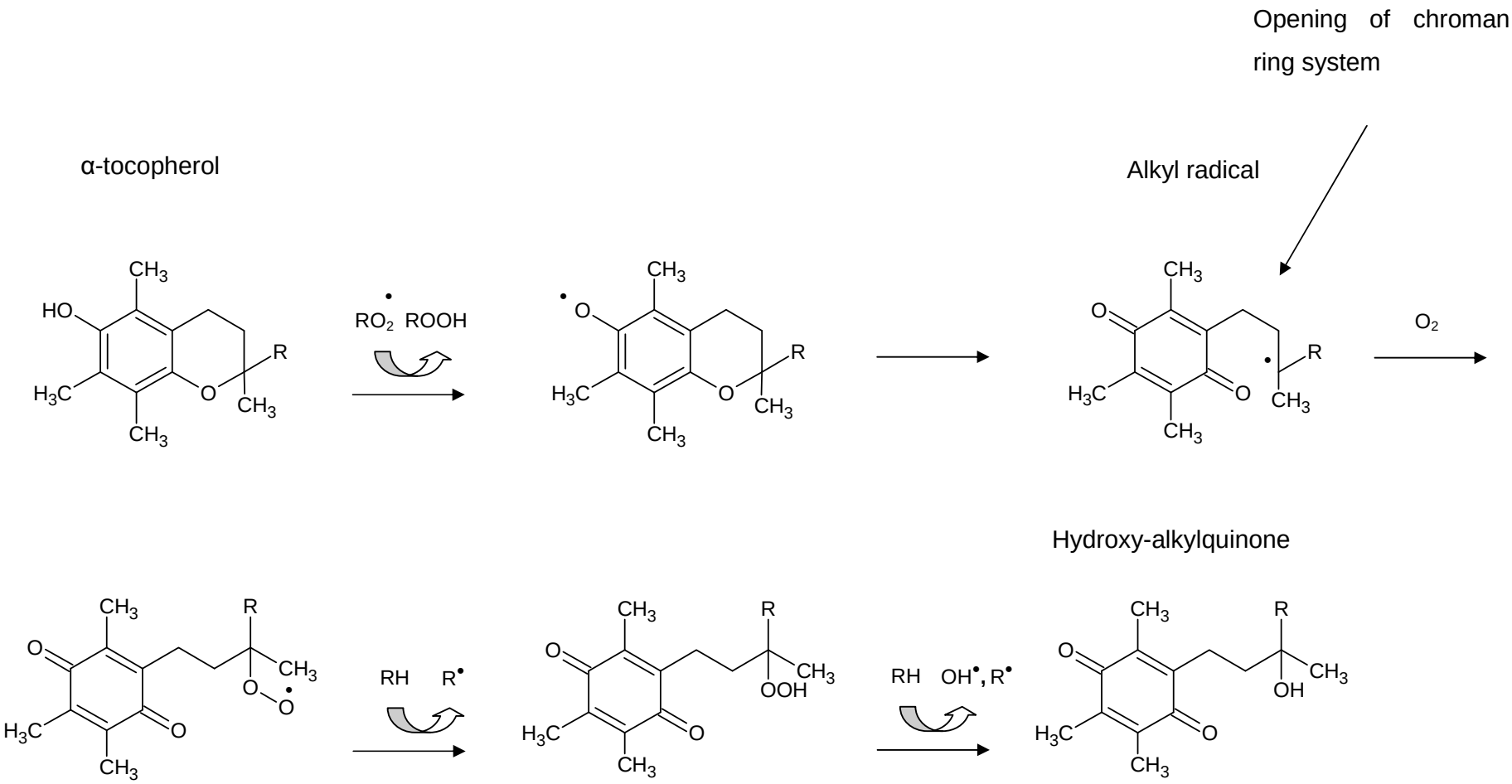


Figure 3.4: Schematic illustration of the oxidative deterioration of α-tocopherol (Belitz *et al*., 2004[a])

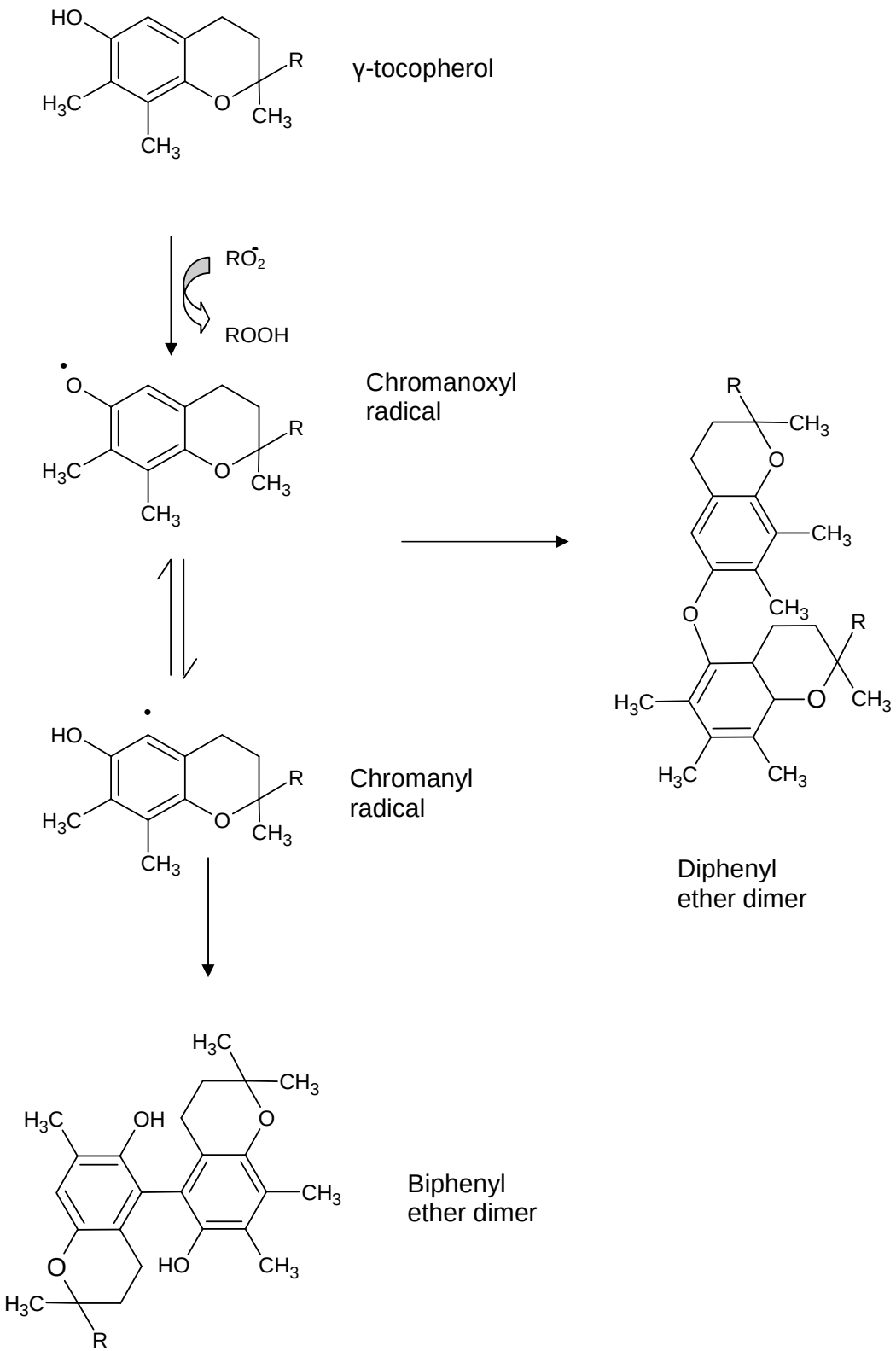


Figure 3.5: Schematic illustration of the oxidative deterioration of γ-tocopherol (Belitz *et al*., 2004[a])

The chromanoxyl radical is stabilised by resonance (Figure 3.6), where the orbital of the 2p electron pair of the ether oxygen and the half occupied molecule orbital of the radical oxygen are aligned parallel to each other (Belitz *et al*., 2004[a]).

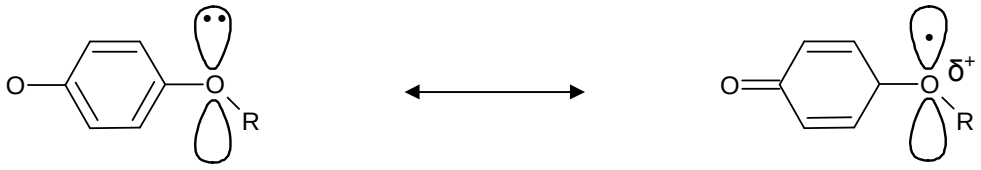


Figure 3.6: Stabilising of the chromanoxyl radical by resonance

Recombination of a chromanoxyl and chromanyl radical results in diphenylether and recombination of two chromanyl radials results in the formation of a biphenyl dimer. These two dimer structures possess one or two phenolic groups that are antioxidatively active. This is in contrast with the opening of the chroman ring that takes place during the formation of the alkyl radical or *p*-quinone (Figure 3.4). Both these breakdown processes, however, lead to the formation of products other than tocopherol, which subsequently lowers the levels of tocopherols measured in a specific sample.

Ripe fruit have higher lipoxygenase content than unripe fruit (Prusky *et al*., 1985; Karni *et al*., 1989). Greater breakdown of tocopherols by lipoxygenase would be expected in ripe fruit leading to lower levels of tocopherols in oil from ripe fruit. In addition, relatively lower temperatures used during freeze-drying would also be expected to preserve the lipoxygenase enzyme while higher temperatures used during oven-drying could inactivate it. Lipoxygenase enzymes extracted from tomatoes proved to have the highest enzyme activity at 25℃, with only 14 % activity remaining at 45℃ (Yilmaz, 2001). Lipoxygenase enz ymes also have a high reaction rate in the temperature range of 0 – 20^{o}C (which are the prevailing temperature conditions during freeze-drying). Thus, relatively higher lipoxygenase levels in ripe fruit, coupled with its preservation during freeze-drying would mean

greater breakdown of tocopherols, hence the observed lower levels in oil from ripe, freeze-dried fruit.

As mentioned earlier, the sterol profile observed for the avocado oil in this work was similar to that reported for other oils, where β-sitosterol is generally the most abundant sterol homologue while campesterol and stigmasterol are usually present in significant levels (Shahidi & Senanayake, 2006).

The observation that campestanol was only detected in oil from ripe, oven-dried fruit suggests that it may have been formed from campesterol possibly due to the action of reductase enzymes. These enzymes appear to have been induced into action by the warm temperature conditions of the oven which may possibly explain the detection of campestanol in oil from ripe, oven-dried fruit and not in oil from freeze-dried fruit. This has been explained earlier in Chapter 2.3, page 103.

The levels of total sterols did not vary significantly between the different treatments. However, when the individual effects of the variables were assessed, a significant effect ($p = 0.027$) (Table 3.1) was observed for drying method. When the average total sterol value was determined for the four fractions, oil from the oven-dried samples had higher total sterol values (4055.60 mg/ kg oil for oil from unripe fruit and 3958.13 mg/ kg oil for oil from ripe fruit) than oil from the freeze-dried samples (3670.28 mg/ kg oil for oil from unripe fruit and 3538.00 mg/ kg oil for oil from ripe fruit). Sterols are not degraded by lipoxygenases (Zhang, Nguyen, Paice, Tsang, & Renaud, 2007) and therefore the lower sterol content of oil from freeze-dried samples cannot be attributed to the fact that lipoxygenases might have been preserved during freeze-drying. Sterols could, however, be oxidised in freeze-dried samples. Freeze-dried samples tend to be more porous and have a more open structure compared to oven-dried samples (Berlin *et al*., 1996). It will therefore be easier for oxygen to penetrate into oil cells of freeze-dried samples and bring about degradation of sterols. Sterols could also have been protected against oxidation in oven-dried samples by the possible formation of compounds with antioxidant activity during oven-drying.

Fruit ripeness and drying method had significant effects on carotenoid levels in the oil both individually and in combination (Table 3.1). Oil from ripe fruit had lower carotenoid levels compared to oil from unripe fruit. Like tocopherols, carotenoids can be broken down by free radicals, created by the action of lipoxygenase enzymes (Lee & Min, 1990) on polyunsaturated fatty acids. Lipoxygenases can co-oxidise carotenoids and chlorophyll and thus degrade the pigments to colourless products (Belitz *et al*., 2004[a]).

Carotenoids act as antioxidants by quenching singlet oxygen and free radicals (Lee & Min, 1990). Carotenoids react with peroxyl or alkoxyl radicals to form resonance-stabilised carbon-centered radicals (Palozza & Krinsky, 1992). It also competes with fatty acids in reacting with free radicals produced by lipoxygenase (Burton, 1989). The carotenoid radicals can be removed from the system by reacting with another peroxyl radical to produce inactive products. The overall effect is to divert a potentially damaging lipid chain reaction into a much less deleterious side reaction involving carotenoids, though this inevitably leads to a reduction in carotenoid levels. Relatively higher levels and activity of lipoxygenase are present in ripe avocado fruit compared to unripe fruit (Prusky *et al*., 1985; Karni *et al*., 1989). This could result in greater breakdown of carotenoids in ripe fruit leading to lower levels of carotenoids in the oil obtained from this fruit.

Relatively lower carotenoid levels were observed in oil from freeze-dried samples of ripe fruit compared to oil from ripe, oven-dried fruit. However, the opposite was observed for unripe fruit where oil from freeze-dried samples had higher carotenoid levels than from oven-dried fruit. In ripe fruit, the lipoxygenase enzyme activity is preserved due to lower temperatures during freeze-drying while the enzyme is inactivated due to the higher temperatures to which it is exposed during oven-drying. This could have resulted in greater breakdown of carotenoids due to the action of lipoxygenase in ripe freeze-dried fruit leading to lower carotenoid levels in the oil. In unripe fruit, relatively lower lipoxygenase levels and activity means that the direct effect of heat and oxidising conditions on carotenoids may be more important. Heat and oxygen promotes the oxidation of unsaturated lipids and the formation of free

radicals which could oxidise carotenoids (Gregory, 1996). In addition, carotenoid molecules undergo some isomerization during the initial steps of degradation during heat treatment (Gregory, 1996; Chen & Chen, 1993). Relatively lower temperature conditions during freeze-drying of unripe fruit may slow down carotenoid breakdown leading to higher levels in the oil compared to oil from unripe, oven-dried fruit.

As observed for the carotenoids, fruit ripeness and drying method (individually and in combination) had significant effects on chlorophyll levels in the oil (Table 3.1). Oil from oven-dried fruit had higher chlorophyll values than oil from freeze-dried fruit. During oven-drying, the effect of heat leads to the conversion of chlorophyll to pheophytin by removal of the Mg^{2+} atom from the tetrapyrrole ring (Figure 3.7) as has been found for broccoli juice (Weemaes, Ooms, Van Loey & Hendrickx, 1999), canola oil (Ward, Scarth, Daun & Thorsteinson, 1994), olive oil (Psomidou & Tsimidou, 2000), sweet potato leaves (Chen & Chen, 1993) and various teas (Suzuki & Shioi, 2003); thus increasing the absorption at 667-669 nm. Chlorophyllase enzyme, heat or acids are usually involved in this process (Von Elbe & Schwarts, 1996). The AOCS method Cc 13i-96 used in this study expresses the content of chlorophyll pigments in terms of pheophytin equivalents (mg pheophytin *a* per kg oil). The higher temperature conditions prevailing during oven-drying would lead to higher conversions of chlorophyll to pheophytin in oven-dried fruit compared to freeze-dried fruit. As a result, higher levels of chlorophyll would be assayed in oil from oven-dried samples compared to freeze-dried samples as observed in this study.

A second method of chlorophyll degradation, namely oxidative cleavage (bleaching) occurs due to the action of pheophorbidase, but enzymes like lipoxygenase, chlorophyll oxidase and peroxidase have also been reported to be involved in chlorophyll bleaching (Orthoefer & Dugan, 1973). Lipoxygenase would be expected to retain its activity during freeze-drying and bring about oxidative bleaching of chlorophyll through the cleaving of the tetrapyrrole ring (Martinez & Labuza, 1968; Martinez, Civello, Chaves & Añón, 2001), thus reducing its levels.

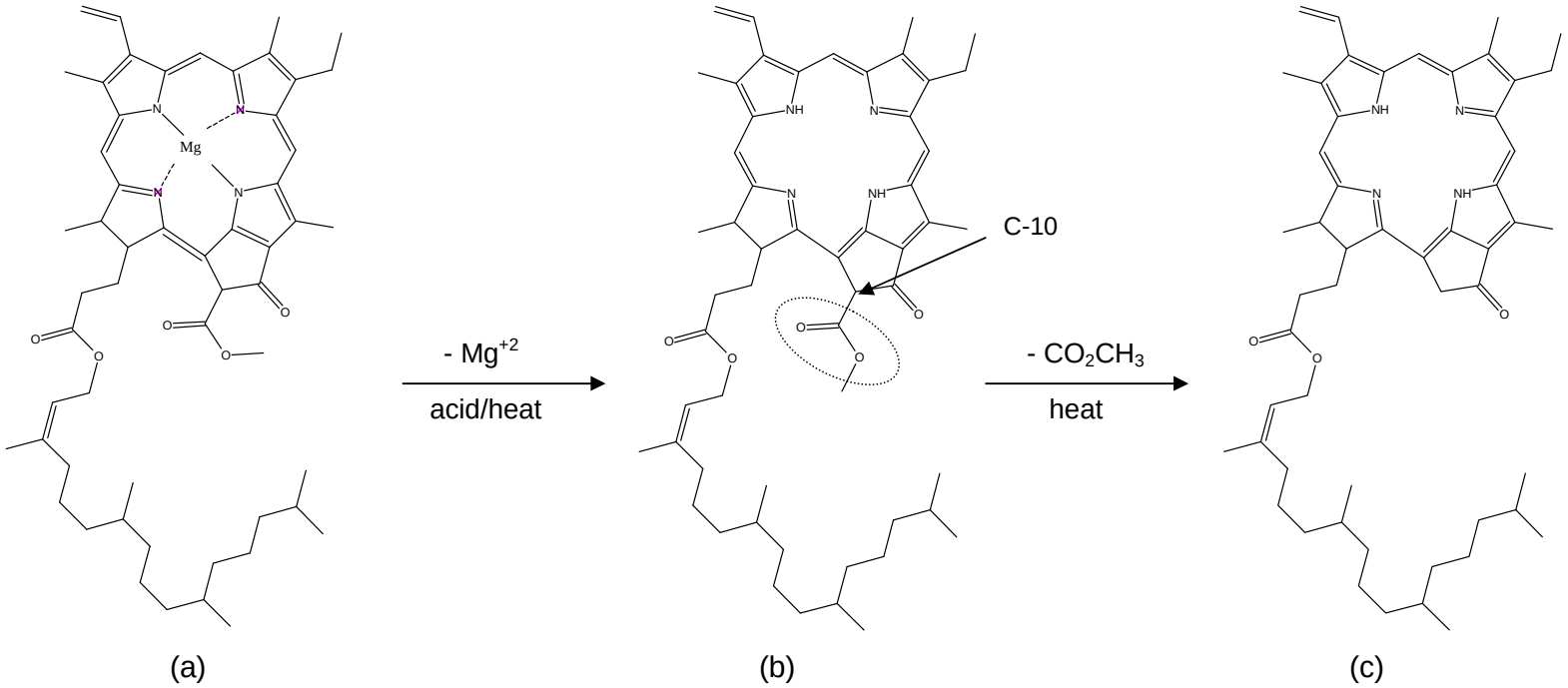


Figure 3.7: The thermal degradation process of (a) chlorophyll *a* to (b) pheophytin *a* and (c) pyropheophytin *a*.

In addition, the greater surface area and porosity of freeze-dried products than those of oven-dried products (Berlin *et al*., 1996; King *et al*., 1986) might have caused higher oxygen permeability, creating more free radicals (Martinez & Labuza, 1968) and subsequently higher oxidative breakdown and lower chlorophyll values for oil extracted from freeze-dried fruit.

Oil from ripe, oven-dried fruit had relatively lower levels of chlorophyll (average of four fractions: 148.03 mg/ kg oil) than oil from unripe, oven-dried fruit (average of four fractions: 178.13 mg/ kg oil). In the same way, oil from ripe, freeze-dried fruit had relatively lower chlorophyll levels (average of four fractions: 96.30 mg/ kg oil) than oil from unripe freeze-dried fruit (average of four fractions: 112.61 mg/kg oil). Similar results were obtained by Ashton *et al*. (2006) who reported a decrease in chlorophyll content of avocado fruit with ripening. Chlorophyll decomposition during senescence and fruit ripening decreases its content (Belitz *et al*., 2004[b]). The increase of oxidative enzymes during fruit ripening (Prusky *et al*., 1985; Karni *et al*., 1989), could lead to oxidative breakdown of chlorophylls, subsequently resulting in lower chlorophyll levels in ripe fruit. Sinnecker, Braga, Macchione, Lanfer-Marquez, (2005) also observed reduced chlorophyll levels in soybean caused by bleaching during air drying at 25℃, while an increase in the levels of pheophytin was observed after oven drying at 75℃.

The colour values are related to the pigments occurring in the oil (Norris, 1982). In general, blue, red and yellow values were higher for oil from oven-dried than freeze-dried fruit (Table 3.2). This could be related to the structural changes that chlorophyll and possibly other pigments not analysed in this study had undergone during oven-drying. Especially oil from ripe, freeze-dried fruit had the lowest average values from the four fractions combined for blue and red. The destruction of the chlorophyll and carotenoids (and possibly other pigments present in the oil) by the action of lipoxygenase in the ripe, freeze-dried fruit could be responsible for the observed low blue and red colour levels in these samples.

Table 3.2: Average lovibond colour values (four fractions combined) of avocado oil extracted from unripe and ripe avocado fruit with supercritical carbon dioxide (SC-CO_2)

Sample	**Blue**	**Red**	**Yellow**
Unripe, freeze-dried	1.33	3.14	22.23
Unripe, oven-dried	2.11	3.30	26.49
Ripe, freeze-dried	1.10	1.83	22.59
Ripe, oven dried	2.50	3.38	26.33

No significant effect on unsaponifiable content was observed for fruit ripeness or drying method (Table 3.1). As mentioned in chapter 2 (p 97) it was expected that the unsaponifiable content of oil from oven-dried avocado, would be higher than that of oil from freeze-dried avocado due to the expected formation of a class of furane compounds during oven-drying (Farines *et al*. 1995). However the amount of unsaponifiable matter in oil extracted from oven-dried fruit did not differ significantly from that of oil extracted from freeze-dried fruit in this study. The conditions used by Farines *et al*. (1995) namely 80℃ for 24 hours was duplicated in this study. Farines *et al*. (1995) extracted the oil containing the furane compounds with hexane. Hexane has a lower selectivity for micro-components (Gómez & de la Ossa, 2002) and it might be possible that these compounds may have been formed, but were not extracted with the SC-CO_2 due to the higher selectivity of SC-CO_2 for micro-components.

It was hypothesized earlier in this study, that the extraction profile of the micro-components would be dependent on the location of micro-components within the cellular structure. This was observed in the current study. This observation can be compared to other studies (Przybylski *et al.* 1998), with pigments eluting later during extraction with SC-CO_2 due to their location within chloroplasts and chromoplasts which have complex membrane systems. The increase of these pigments may, however, be noted at different stages and levels during progressive extraction in different studies due to the variation in pressure, flow rates and particle size used.

Micro-components which eluted later in the current study might have eluted sooner in the study conducted by Przybylski *et al.,* (1998). This might have happened because extraction was conducted at higher pressures and flow rates by these authors. These factors might have caused pigments to be liberated from their rigid structures earlier during the extraction. The generation of heat in high pressure pumps which leads to variation in flow rate influenced the consistency of the extraction rates in this study. It was therefore possible to compare extraction trends, but the exact volume of CO_2 needed to elute a certain concentration of micro-components was difficult to compare to literature.

The total unsaponifiable levels decreased with progressive extraction (Figure 2.3.5, p. 97). The levels of total unsaponifiables are a resultant of the levels of its individual components (carotenoids, chlorophyll, tocopherols, sterols and other hydrocarbons not determined in this study). When the analytically determined unsaponifiable fraction was compared to the calculated unsaponifiable fraction (the total of the individual micro-components measured in this study), there seemed to be some components missing from the calculated values. The calculated values were all below 1 g/ 100 g while the analytically determined values ranged between 2 and 5 g/ 100 g oil. This may be attributed to the presence of components that were not measured individually, but made up part of the total unsaponifiable fraction determined analytically.

The avocado skin was not removed before oil extraction. Cutins and waxes occur on the epidermal cells of fruit (Mauseth, 1995). Waxes are non-polar (Gunstone, 1996) and would be highly soluble (Gunstone, 1996) in the non-polar SC-CO_2 (Jikei *et al*., 2006). Because these compounds are located on the surface of the skin, they would be the first compounds to be extracted with the oil. High levels of waxes in the beginning of the extraction could explain the higher masses obtained for the analytical determination of the unsaponifiable fraction of the earlier oil fractions.

There was a general decreasing trend with progressive extraction for total tocopherol levels of oil extracted from avocado fruit exposed to all treatments,

except for oil from ripe, freeze-dried fruit. No clear and consistent trends were distinguishable for the different isomers, although δ-tocopherol seemed to decrease with progressive extraction.

Levels of total sterols remained unchanged with progressive extraction (Table 3.1). Although the effect of progressive extraction was significant for some of the individual sterols, the trends were not uniformly increasing or decreasing and no definite pattern could be distinguished for sterol elution with progressive extraction.

The difference in the effect of progressive extraction on the pigments (chlorophyll and carotenoids) compared to the other micro-components (tocopherols and sterols) was unambiguous. Both chlorophyll (Figure 2.3.2, p. 91) and carotenoids (Figure 2.3.4, p. 95) increased significantly with progressive extraction. This was confirmed in the colour determination. The lovibond values red and blue increased with progressive extraction, while yellow did not fluctuate except for an increase in that of oil from ripe, freeze-dried fruit. Przybylski, Lee & Kim (1998) have also reported increases in lovibond blue and red colour values for canola oil extracted with SC-CO_2. The carotenoid and chlorophyll content correlated well with the red (0.85 and 0.92 respectively) and blue (0.82 and 0.96 respectively) lovibond colour determinations. This indicated that the colour measurement was a reflection of these two micro-components.

It seems like the actual location of the colour pigments within the plant material, could have been responsible for the increase in pigment (carotenoids and chlorophyll) content with progressive extraction. SC-CO_2 is a non-polar solvent (Jikei *et al*., 2006) and one would expect more non-polar compounds to elute first during extraction. Chlorophyll is a more polar molecule than the carotenoid group (Macías-Sánchez, Mantell, Rodríguez, Martínez de la Ossa, Lubián, & Montero, 2005), yet the concentration of both were much higher in the latter oil fractions, which could indicate that the increase in yield of these components must have been linked to their position within the plant material. Both these pigments are located inside chloroplasts and chromoplasts within the parenchyma cells (Mauseth, 1995)

and some of the carotenoids may be concentrated in the idioblast cells (Platt & Thompson, 1992). All of these structures have thickened cell walls, while the large parenchyma cells have thinner cell walls (Mauseth, 1995). When pressure is applied during SC-CO_2 extraction, the parenchyma cells are most likely to rupture first, allowing the extraction of the triglycerides. When these cells have been depleted of oil, the pressure would then be applied to the chloroplasts, chromoplasts and idioblast cells, which presumably would have remained intact during the first stages of extraction. These structures would then start to rupture as they retain less of their structural integrity, thus releasing the pigments more in the latter oil fractions. This hypothesized process is illustrated in Figure 3.8.

Sterols and tocopherols, on the other hand are located in cell membranes (Kumar *et al*., 2005; Taiz & Zeiger, 2006) which could explain the more equal distribution of these compounds throughout progressive extraction. Cell membranes consist of lipid bilayers with sterols and tocopherols embedded in them.

These structures are not rigid and do not have multiple layers like thylakoid cells (where chlorophyll is located), and may therefore be more penetrable by the SC-CO_2 (Garret & Grisham, 1995; Mauseth, 1995). The levels of tocopherols and sterols would therefore not be expected to vary too much with progressive extraction.

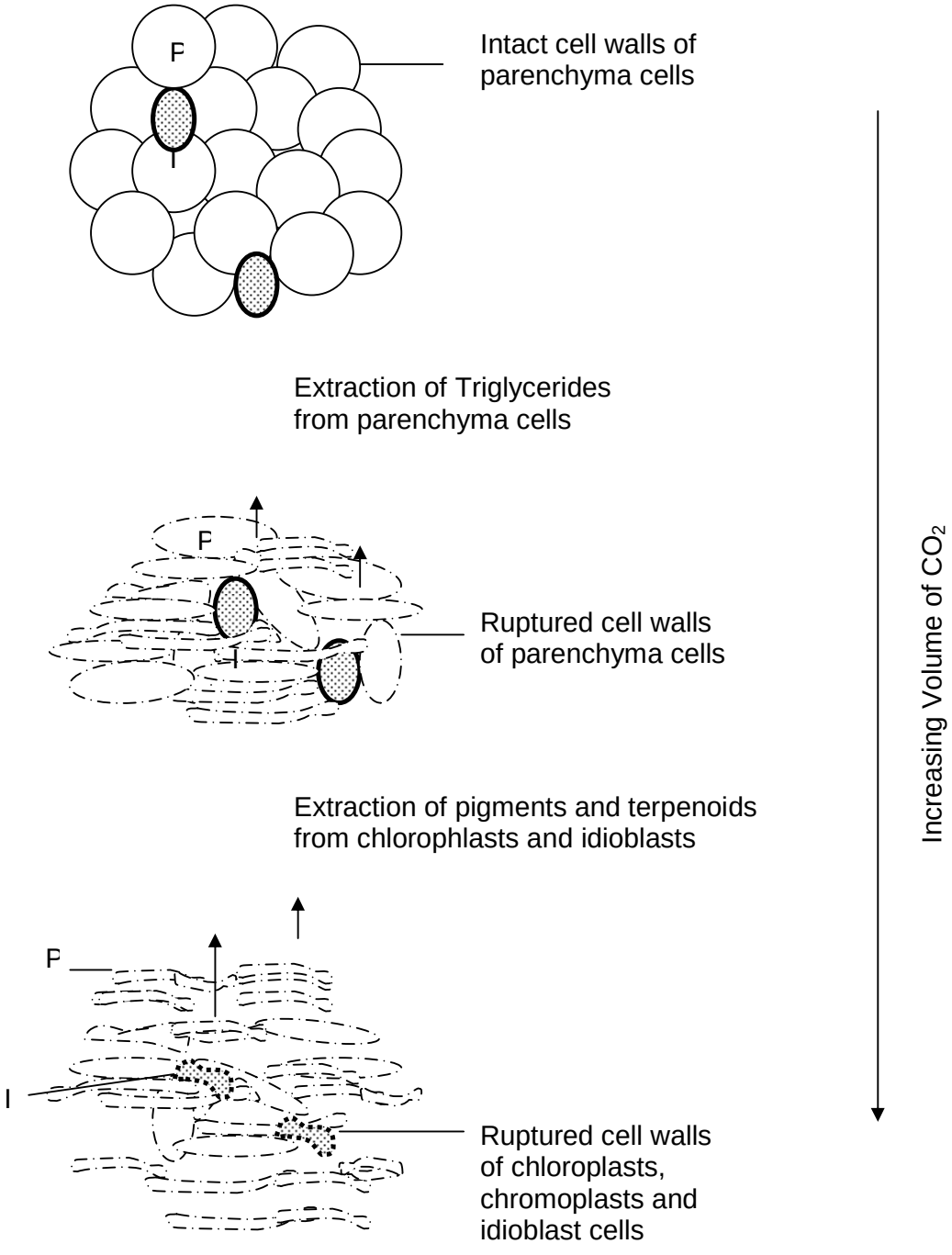


Figure 3.8: Figures illustrating the destruction of cellular integrity during progressive extraction with SC-CO_2 indicating how triglycerides were probably extracted first, followed by pigments and terpenoids contained within more rigid structures.

3.3 Oxidative stability of avocado oil

The fatty acid profile or the saponifiable fraction of oil is normally the first indication of the oxidative stability of the oil (Sonntag, 1997[b]). Based on fatty acid profile (macro-components) alone, monounsaturated oils like avocado and olive oil are oxidatively more stable than polyunsaturated oils like sunflower oil. Other factors like the micro-components, must, however, be taken into account when considering the oxidative stability of oil.

In this study, neither the degree of ripeness nor the drying method of the fruit had any significant effect on the fatty acid profile of the avocado oil (Table 3.1). This suggests that any changes in oxidative stability of the oil due to fruit ripeness or method of fruit drying may be due more to other factors such as micro-component content rather than fatty acid profile. Progressive extraction did, however, influence the extraction profile of the oil and it was observed that higher levels of polyunsaturated fatty acids, linoleic and linolenic acids eluted during the beginning of extraction than the latter fractions. Higher levels of oleic acid eluted towards the end of the extraction.

In this study, various oil oxidative stability parameters, namely, peroxide value (PV), anisidine value (AV), total oxidation (TOTOX), free fatty acids (FFA) and oxidative stability index using the Rancimat (OSI) were used to evaluate the oxidative stability of the avocado oil. The drying method of the fruit had significant effects on the PV, AV and TOTOX values of the oil samples (Table 3.1). The PV, AV and TOTOX indicated that oil extracted from oven-dried fruit had undergone more oxidative deterioration than oil extracted from freeze-dried fruit. Oil from oven-dried fruit had low PVs but high AVs, while oil from freeze-dried fruit had high PVs but low AVs. Oil from oven-dried fruit also had a higher mean TOTOX value than oil from freeze-dried fruit. These observations suggest that the rate of oxidation was higher for oil from oven-dried fruit than from freeze-dried fruit.

The production of primary oxidation products (measured by the PV) and secondary oxidation products (measured by the AV) during the course of oil oxidation may be

represented as shown in Figure 3.9 below. During the early stages of the oil oxidation process, primary oxidation products (peroxides) are produced and therefore the PV increases gradually. At a certain maximum value of the PV these primary oxidation products begin to break down to form secondary oxidation products at which point AV values begin to increase. The PV curve reaches a turning point (as shown in Figure 3.9) and the PV values begin to decrease. This turning point differs for different oils (Rossell, 1994).

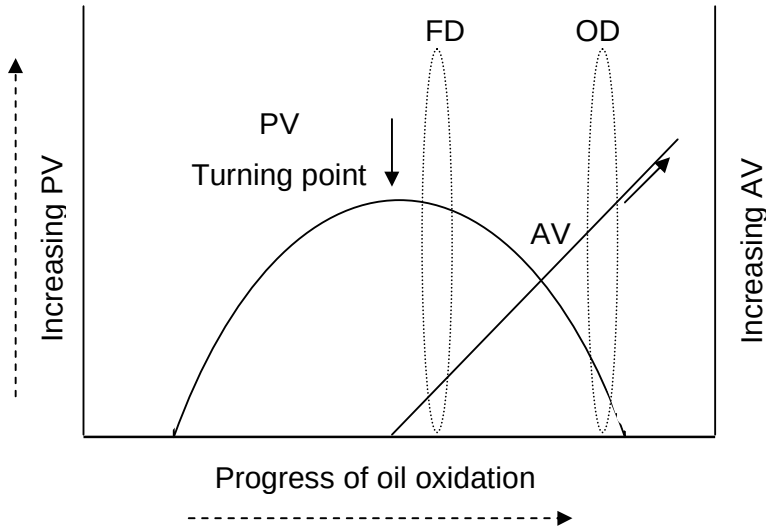


Figure 3.9: Typical peroxide value (PV) and anisidine value (AV) curves depicting the different oxidation rates of oil extracted from freeze-dried (FD) and oven-dried (OD) fruit.

Oil from freeze-dried fruit had high peroxide values but low anisidine values. This suggests that the oil was still in the early stages of oxidative deterioration, probably just past the turning point, where peroxides were starting to break down to secondary products (Figure 3.9). In contrast, oil from oven-dried fruit, had relatively lower peroxide values and higher anisidine values, indicating that this oil was in the later stages of oxidative deterioration. The PV had already reached the point where it started to decrease because many of the peroxides had been broken down to secondary oxidation products (aldehydes) measured by the anisidine value.

Oxygen and heat are the two main factors affecting oxidative deterioration in oils (Hamilton, 1994). These factors are not present during freeze-drying as the process is executed at low temperatures under vacuum. The only times when these factors were present, was during sample preparation and storage and perhaps when the plates were heated to approximately 25℃ during fre eze-drying. These conditions are less favourable for oxidative deterioration than the conditions during oven-drying. During oven-drying samples were exposed to temperatures of 80℃ in a forced draft oven, exposing it to both oxygen and high temperatures. The oxidative deterioration would therefore be more advanced in the oven-dried samples with higher amounts of secondary products being detected in these oils.

Fruit ripeness had significant effects on the PV, AV and TOTOX values of the oil (Table 3.1). The effect of fruit ripeness was clearly noticeable in the PVs of oil from freeze-dried samples where oil from ripe, freeze-dried fruit had higher PVs compared to oil from unripe, freeze-dried fruit. Ripe fruit have higher levels of enzymes such as lipases and lipoxygenases (Pesis *et al*., 1978; Platt & Thomson, 1992; Prasanna *et al*., 2007) and presumably higher activity than unripe fruit. These enzymes will also be preserved under the low temperature conditions of freeze-drying compared to oven-drying. For ripe, freeze-dried fruit therefore, there will be higher release of free fatty acids from triglyceride molecules due to the action of lipases. These free fatty acids would then be degraded into peroxides due to the action of lipoxygenase leading to the observed high PVs for oil from ripe, freeze-dried fruit.

The free fatty acid content (FFA), which is an indication of the hydrolytic deterioration, was the highest in samples from ripe, freeze-dried fruit compared to oil from all the other treatments. This can also be related to the higher content of lipases present in ripe fruit, with preserved activity due to freeze-drying.

Fruit ripeness and method of drying also had significant effects on the OSI of the oil (Table 3.1). Oil from ripe, freeze-dried fruit had the lowest OSI of all the treatments. This was in agreement with the observed relatively higher levels of hydrolytic

deterioration (high FFA) of these samples due to preserved enzyme activity under the lower temperature conditions of freeze-drying as explained earlier. Interestingly, oil from oven-dried fruit generally had higher OSI than oil from freeze-dried fruit. This was in contradiction with the observations from the PV, AV and TOTOX results. Although the oil from oven-dried fruit generally had high AVs and high TOTOX values, indicating advanced oxidative deterioration, their corresponding OSI values were also high, meaning they were oxidatively stable. On the other hand, though oils from freeze-dried fruit had relatively lower AVs and TOTOX values, which may suggest low oxidative deterioration, their corresponding OSI values were relatively lower. These observations suggest that some antioxidant compounds could have been formed during the oven-drying process which conferred high OSI to the oil.

The relatively higher levels of unsaturation in the first oil fractions could have contributed to the lower oxidative stability of the oil in these fractions. The concentrations of the C18:2 and C18:3, were however, low in comparison to the C18:1, which is the main fatty acid present in avocado oil and it is questionable whether the variation between the first and last fractions for C18:2 could have had a meaningful effect on the oxidative stability of the oil. The relative increase in monounsaturated versus polyunsaturated fatty acids in the last fractions, could, however have made a small contribution to the increased oxidative stability of the last oil fractions. This is supported in the positive correlation between the OSI and oleic acid levels (0.68) and the smaller, yet significant correlation between the OSI and linoleic acid (-0.53).

Progressive extraction had significant effects on all the oxidative stability parameters, namely PV, AV, TOTOX, FFA and OSI (Table 3.1). Although the PV, AV and TOTOX values of the oil changed with progressive extraction, these changes were not uniform and did not follow a specific trend. It could be argued that for the PV, AV and TOTOX values, perhaps the effects of fruit ripeness and drying method may be of more significance than the effect of progressive extraction in this study.

The FFA values all decreased with progressive extraction (Figure 2.2.7, p. 68). Similar results were reported by Przybylski, Lee & Kim (1998), who observed a decrease in FFA levels during progressive extraction of canola oil with SC-CO_2. This could be because many free fatty acids were located on the surface of the ground avocado fruit material prior to extraction due to hydrolytic deterioration of the oil on the surface of the plant material during processing. These free fatty acids would elute first due to their availability to the solvent as well as their solubility in the non-polar SC-CO_2 due to their non-polar nature, and their concentration would decrease as the extraction progresses.

Przybylski, Lee & Kim (1998) also reported higher rates of elution of polyunsaturated fatty acids at the beginning of extraction. Polyunsaturated fatty acids oxidise more readily than monounsaturated and saturated fatty acids. They attributed the lower oxidative stability of the first fractions to the higher FFA content, higher amount of polyunsaturated fatty acids and absence of phospholipids in the first fractions. Phospholipids were not determined in this study.

In this study, the OSI increased with progressive extraction in opposite fashion to the FFA. This may be explained by the fact that higher FFA values in the earlier fractions indicating higher hydrolytic deterioration (higher release of free fatty acids) could lead to lower OSI in these fractions due to higher susceptibility of these fatty acids to oxidative deterioration. This was in accordance with the study conducted on SC-CO_2-extracted canola oil by Przybylski, Lee and Kim (1998), which also showed lower FFA values and higher oxidative stability for later oil fractions. However, as alluded to earlier, the high OSI values in the later fractions did not correspond with the high levels of oxidative deterioration, specifically AV, observed in these fractions.

As expected, the correlation between the FFA value and the OSI value for avocado oil from all samples was negative (-0.69). The factors influencing hydrolytic deterioration of oil are heat, moisture and enzymes (Hamilton, 1994). High FFA

values indicate increased hydrolytic deterioration, which creates more substrate for lipoxygenase enzymes and in turn, decreases the OSI (Hudson & Gordon, 1994).

There was an obvious contradiction between the high oxidative deterioration values measured for the oil and the high OSI values measured for the same samples of avocado oil. It was expected that oil with high levels of oxidative deterioration as measured by the PV and AV and calculated by the TOTOX would have a relatively lower oxidative stability. In fact, the correlation between the AV and OSI for the oil was positive (0.56). The factors which influence oxidative deterioration in oil, measured by the AV, are heat and oxygen (Hamilton, 1994). As relatively lower temperatures are applied under vacuum during freeze-drying, the factors that could influence oxidative deterioration are minimal under these conditions. These factors, however, are both present during oven-drying in a forced draft oven where high temperatures and air containing oxygen are applied to the samples. Correlations were re-calculated for oil from freeze-dried and oven-dried fruit separately to investigate reasons for the positive correlation between the OSI and AV.

For oil from freeze-dried fruit, the correlation between the FFA and OSI became more negative (-0.84), while the correlation between the OSI and AV also became negative (-0.74). During the process of oxidative and hydrolytic oil degradation, one would expect the OSI of the oil to decrease as the FFA and AV of the oil increases. Increased FFA values in especially oil from ripe, freeze-dried fruit can be related to enzyme activity (lipase) which was preserved due to low temperatures during freeze-drying, causing the hydrolysis of fatty acids from glycerol (Hamilton, 1994). Free fatty acids, in turn, would be more rapidly oxidised by lipoxygenase enzymes which would also have been preserved during freeze-drying, causing the formation of hydroperoxides and in time, aldehydic compounds measured by the AV (Rossell, 1994). Therefore the correlations between FFA and OSI and AV and OSI for oil from freeze-dried fruit were reasonable and in agreement with the laws of oil oxidation. In further confirmation of this, the predictive models developed for oil from freeze-dried fruit selected the FFA value as the most important variable in predicting the OSI of the oil.

For oil from oven-dried fruit, the correlation between FFA and OSI remained negative (-0.73) and the correlation between the AV and OSI became notably more positive (0.73). The negative correlation between the FFA value and OSI was expected as this conformed to oil oxidation chemistry as discussed above, where oil containing higher amounts of FFA usually has shorter induction periods (Hudson & Gordon, 1994).

However, the positive correlation between the AV and OSI for oil from oven-dried fruit was anomalous. This was confirmed by the predictive model developed for oil from oven-dried fruit, which selected the AV as the most important variable in predicting the OSI of the oil. This observation suggests the possible formation of compounds with antioxidant potential by the same factors which are responsible for increasing the AV of the oil. These compounds with antioxidant potential would then confer high OSI to oil from oven-dried fruit notwithstanding the high AV of this oil. In an attempt to explain this, the relationships between the micro-components determined in this study and the OSI were investigated.

Significant positive correlations were found between some of the micro-components in the oil and the OSI. There were positive correlations between the OSI and chlorophyll (0.83), carotenoids (0.80) and δ-7 stigmasterol (0.62), indicating that these micro-components exerted antioxidant effects in the oil. Positive correlations were also found between the colour pigments blue (0.78) and red (0.83) and the OSI value. As mentioned before, the blue and red value correlated well with the chlorophyll (blue: 0.96; red: 0.92) and carotenoid (blue: 0.82; red: 0.85) values.

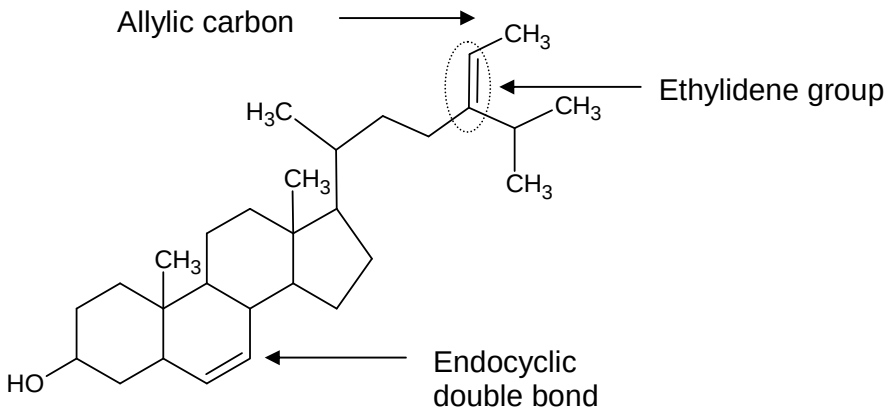
Although the antioxidant effects of tocopherols are well-known (Murcia *et al*., 2001), the only significant correlation between the OSI value and any of the tocopherol isomers, including total tocopherol, was for δ-tocopherol (-0.60) (the most abundant isomer in avocado oil) indicating a pro-oxidant activity. As mentioned earlier, this could be attributed to a decrease in the extraction profile of the δ-tocopherol relative to the other isomers, and could be a chance correlation rather than an indication of

pro-oxidant action of the isomer. As will be discussed later, according to predictive models, however, total tocopherols had a minor, positive effect on the OSI.

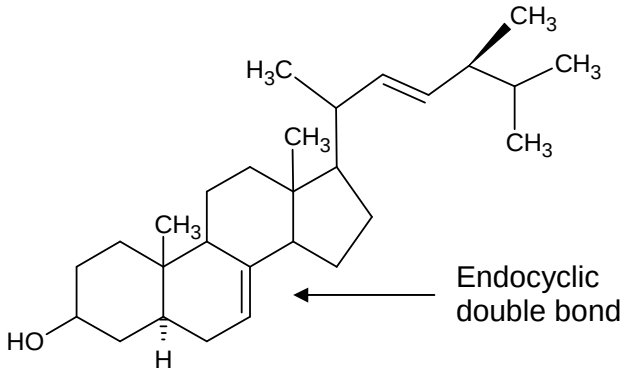
The stabilizing effect of some sterols in oil subjected to prolonged heating have been reported by various authors (Sims *et al*. 1972; Boskou & Morton, 1976; White & Armstrong, 1986). A minor, non-significant positive effect of total sterols on the OSI of extra virgin olive oil have been reported by Cercaci *et al*. (2007). Several sterols including fucosterol, Δ-5 avenasterol, vernosterol, fucosterol and citrostadienol have been shown to have antioxidant activity in oils at 180ºC (White & Armstrong, 1986; Rajalakshmi & Narasimhan, 1995). The presence of a free hydrogen atom on the allylic carbon of the ethylidene group in the side chain of δ-5 avenasterol appears to make it more prone to radical formation (Figure 3.10).

The intensity of the antioxidant effect is furthermore dependent on the number and position of endocyclic double bonds (Cercaci *et al*., 2007). No literature was found on a possible antioxidant effect of δ-7 stigmasterol. No ethylidene group is present in the side chain of δ-7 stigmasterol, which makes it difficult to attribute an antioxidant effect to this sterol. The sterol is, however prone to autoxidation due to its endocyclic double bonds and oxidation of the sterol instead of other lipid molecules (such as triacylglycerols and fatty acids) could protect the lipid molecules from oxidation (Cercaci *et al*., 2007). The levels of δ-7 stigmasterol were, however, relatively low in this study (chapter 2.3, page 102) and the antioxidant effect observed for this sterol might be due to a chance correlation between the OSI and δ-7 stigmasterol content of the avocado oil due to a decrease in the extraction profile of the δ-7 stigmasterol content relative to the other sterols in a similar fashion to that of δ-tocopherol.

Carotenoids act as free radical scavengers in oil and thus inhibit lipid peroxidation (Zambiazi & Przybylski, 1998). Warner & Frankel (1987) reported that the presence of 5 to 20 ppm of β-Carotene had a significant effect in protecting soybean oil against light deterioration.



(a)



(b)

Figure 3.10: Structural differences between (a) δ-5 avenasterol and (b) δ-7 stigmasterol.

Chlorophyll is a strong pro-oxidant in oils. The oxidation reaction, in which chlorophyll acts as oxidising agent, is catalyzed by light (Smouse, 1995). However, a derivative of chlorophyll, pheophytin, has been observed to have antioxidant potential in oil (Psomiadou & Tsimidou, 2002).

Predictive models were developed to determine the influence of the micro-components on the oxidative stability of the avocado oil extracted with SC-CO_2. A predictive model developed including carotenoids, chlorophyll, all of the sterols and all of the tocopherol isomers individually, proved to have too many variables to be realistic. A model using carotenoids, chlorophyll, total sterols and total tocopherols

selected chlorophyll (β = 0.97) to be the most important variable in predicting the OSI of the avocado oil, with an R^2 value of 0.77 (Table 2.3.4, p. 108). The carotenoid value was declared redundant by the program, total sterols were indicated to have a prooxidant effect (β = -0.24) on oxidative stability while tocopherols were indicated to have an antioxidant effect (β = 0.23). Both of these variables had a minor effect relative to that of chlorophyll. A third model, excluding chlorophyll and using total micro-component values, selected carotenoids (β = 0.79) as being the most important variable in predicting the OSI of avocado oil with an R^2 value of 0.63. These two models indicated that chlorophyll and carotenoids were the most important variables influencing the OSI of avocado oil and agreed with the correlations.

The positive correlation between the AV and OSI can therefore be explained by the presence of antioxidant compounds endogenous to the oil, or formation of compounds with antioxidant potential during oven-drying. These compounds may exert strong antioxidant effects and give the oil high oxidative stability despite the apparent presence of secondary oxidation products. As indicated by the correlations and the models, carotenoids and chlorophyll may be such antioxidant compounds. The mechanism by which carotenoids may exert antioxidant effects (by quenching singlet oxygen and reacting with free radicals to form resonance-stabilised carbon-centred radicals) has been explained earlier on in this discussion.

The higher R^2 value (0.77) for the model that selected chlorophyll, indicated that the scatter had a better fit around the line, which means that the predicted values were closer to the observed values than for the model using only carotenoids, suggesting that the chlorophyll might be the more important variable.

As mentioned earlier, chlorophyll is generally considered a pro-oxidant compound in oil (Endo *et al*., 1984; Kritisakis & Dugan, 1985). However, under certain conditions, chlorophyll may be converted into derivatives that have antioxidant effects. The formation of pheophytin from chlorophyll due to heat has been well established (Suzuki & Shioi, 2003). The magnesium atom in chlorophyll is easily displaced by

two hydrogens during heating, resulting in the formation of olive-brown pheophytins (Von Elbe & Schwartz, 1996) (Figure 3.5). During further heating, replacement of the C-10 carbomethoxy group of pheophytin with a hydrogen atom results in the formation of olive coloured pyropheophytin. Conversion of chlorophyll to pheophytin and further on to pyropheophytin could therefore have occurred in the avocado fruit during oven-drying.

Psomiadou and Tsimidou (2002) observed the formation of pyropheophytin *a* in olive oil after oven treatment at 40 and 60℃. A strong antioxidant activity for pyropheophytin *a* has been reported in literature (Cahyana *et al*., 1992). Psomiadou and Tsimidou (2002) showed that pheophytin *a* exerted antioxidant effects in olive oil in a concentration-dependent manner. It seems that the antioxidant activity of pheophytin *a* is enhanced by the presence of pyropheophytin, the formation of which is enhanced at higher temperatures.

The literature provides a somewhat contrasting picture concerning the effect of chlorophyll and its derivatives on the oxidative stability of oils. It has been suggested that chlorophyll exerts pro-oxidant activity under light conditions due to a transfer of the energy of singlet-excited chlorophyll to oxygen that would form reactive species (Endo *et al*., 1985[a], 1985[b]). The same authors reported that chlorophyll and pheophytin provide protection of vegetable oils stored in the dark against autoxidation by a hydrogen-donating mechanism, thereby breaking the radical chain reactions. They also stated that the intact chemical structure of porphyrin seemed to be essential for antioxidant activity. In some studies, chlorophylls have been found to be better antioxidants than pheophytins (Hoshina, Tomita & Shioi, 1998), while others have reported that the presence of chlorophyll in tea extracts was responsible for a pro-oxidant effect on the oxidation of marine oils (Wanasundara & Shahidi, 1998).

Varying results regarding the antioxidant effects of chlorophyll and its derivatives have also been obtained with different antioxidant tests including peroxide and carbonyl value (Endo *et al*., 1985[a]), the β-carotene bleaching method and 2,2-

diphenyl picrylhydrazyl (DPPH) radical scavenging method (Lanfer-Marquez, Barros & Sinnecker, 2005) as well as the ferric thiocyanate and thiobarbituric acid (TBA) method (Cahyana *et al*., 1992). Becker, Nissen, & Skibsted (2004) suggest that some factors responsible for obtaining conflicting results in measurements of antioxidant activity for the same compounds are the physical structure of the test system, the nature of the substrate for oxidation and the analytical method employed. It therefore seems that not a single method is able to offer a comprehensive prediction of antioxidant efficacy of chlorophyll and its breakdown products and that ideally, more than one method should be performed to determine their antioxidant activity.

The results obtained in this study are in accordance with the findings of Psomiadou and Tsimidou (2002) concerning the antioxidative potential of chlorophyll derivatives. Later fractions of the SC-CO_2-extracted avocado oil in this work had longer induction periods which correlated well (0.83) with the chlorophyll (essentially pheophytin *a*) concentrations in the fractions. The oven-drying of the fruit (80℃), which probably led to the production of the pheophytin *a* (Suzuki & Shioi, 2003) could also have caused the production of pyropheophytin *a*, thus enhancing the antioxidant potential of pheophytin *a*.

It can therefore be concluded that the possible formation of pheophytin and pyropheophytin from chlorophyll during oven-drying of the avocado fruit, could have caused the positive correlation between the AV and OSI of the oil extracted from oven-dried fruit because the same factors causing the high oxidative deterioration (namely heat and oxygen), could be responsible for the formation of these compounds with antioxidant potential. It must be borne in mind however, that in the present study, the antioxidant effect observed in the avocado oil was attributed to a combination of factors including carotenoids, chlorophyll and free fatty acid content.

The models developed in this study supported the deduction made from the correlations between the micro-components and the OSI, where chlorophyll and carotenoids had high correlations with the OSI. The models are, however, probably

only applicable to this set of data and the inclusion of more repetitions will expand the applications of the models in future.

The choice between which degree of fruit ripeness and drying method to use for optimum yield, favourable micro-component content and best possible oxidative stability will be to a great extent dependent on the target market. The oil yield is higher from ripe than from unripe avocado fruit. However, the quality of oil extracted from the ripe or unripe fruit is closely related to how the fruit is dried. Athough freeze- drying might result in a higher oil yield, the presence of enzyme activity might lead to hydrolytic and oxidative deterioration and the destruction of valuable micro-components. Oven-drying, on the other hand, leads to a lower oil yield and higher oxidative deterioration, but seems to favour the formation of compounds with antioxidant activity and produces oil with high oxidative stability. The content and effect of these components on the oil might be more significant to the industry than the relatively higher oxidative deterioration associated with the oil produced from oven-dried fruit. The extraction of avocado oil with SC-CO_2 can therefore potentially be used for the production of a niche market product with increased levels of natural antioxidants which could confer higher oxidative stability to the oil and provide potential health benefits to the consumer.

4. CONCLUSIONS AND RECOMMENDATIONS

The extractability or yield of oil from avocado fruit is dependent on ripeness of the fruit, method of drying of the fruit and method of extraction using either hexane or SC-CO_2.

The oil yield from ripe avocado fruit is greater than from unripe fruit. This could be due to the action of enzymes such as cellulases and polygalacturonases which degrade the cell walls of the oil-containing parenchyma cells during fruit ripening, making the oil more available for extraction. Avocado oil is more extractable from freeze-dried avocado fruit than from oven-dried fruit. During oven-drying, gelatinisation and dehydration of starch and protein crosslinking due to relatively higher temperatures may lead to formation of structures that act as physical barriers around oil cells, thus reducing oil extractability. Freeze-drying on the other hand is conducted at lower temperatures, thus formation of structural barriers due to changes in starch and protein is minimal. The freeze-dried plant material is therefore more porous and has higher oil extractability.

Higher oil yields from avocado fruit are obtained with hexane as an extraction solvent compared to SC-CO_2 because hexane permeates the whole plant material, is less selective and will extract higher amounts of lipid soluble material like micro-components (such as tocopherols, sterols, chlorophyll, carotenoids), phospholipids and waxes. On the other hand, high pressures exerted during SC-CO_2 extraction may result in compacting of the plant material and the creation of paths of least resistance. The majority of the SC-CO_2 will move through the paths of least resistance resulting in incomplete extraction in compacted areas and therefore lower oil yield compared to hexane extraction.

It must be borne in mind that preparation of especially ripe avocado fruit for SEM could potentially lead to creation of artefacts. For instance, when the ripe fruit tissue is cut, cell walls may tear during separation and create holes or pits which may be assumed to be formed exclusively from enzyme hydrolysis during ripening. Although SEM serves the purpose of studying the structural differences of the

avocado mesocarp before and after pre treatment and extraction, light microscopy may be a more informative technique due to its potential to visually show the amount of oil retention in the cells.

Sample preparation for oil extraction such as prior deep-freezing of avocado fruit samples could bring about some oil losses during the oven-drying process. Large ice crystals are formed during freezing which damage the cells and they lose turgidity during thawing in the oven. This facilitates oil leakage from the damaged cells in the warm temperature conditions of the oven. Rapid freezing techniques like cryogenic freezing could be used in a future study to eliminate the structural damage caused by the formation of large ice crystals during deep-freezing.

As expected, oleic acid was the most abundant fatty acid in the avocado oil. Degree of ripeness and drying method did not influence the fatty acid profile. However, progressive extraction increased the oleic acid but decreased the linoleic acid. Overall the fatty acid profile did not have a great effect on the OSI in comparison with some of the micro-components.

Oxidative deterioration occurs at a higher rate in avocado oil extracted with SC-CO_2 from oven-dried fruit than in oil from freeze-dried fruit. This is because the prevailing conditions in the oven namely, high temperatures and constant air movement (presence of oxygen), which favour oxidative deterioration of oils are present in the hot air oven, while vacuum (no oxygen) and lower temperatures are present in the freeze-dryer.

Hydrolytic deterioration is greater in SC-CO_2-extracted oil from ripe, freeze-dried fruit than in ripe, oven-dried fruit because the activity of lipase enzymes present in ripe fruit is preserved at low temperatures in the freeze-dryer while they are inactivated at higher temperatures in the oven. Hydrolytic deterioration is less pronounced in oil from unripe fruit compared to ripe fruit, most likely due to the relatively lower levels of lipase enzymes in unripe fruit. The free fatty acid content is higher during the first

stages of progressive extraction because they are more available and soluble in the SC-CO_2. This contributes to the lower oxidative stability in the first fractions.

Oil from ripe, freeze-dried avocado has relatively lower levels of chlorophyll, carotenoids and tocopherols, than oil samples from the other treatments. Lipoxygenase levels increase during ripening of avocado and will lead to higher oxidation of chlorophyll, carotenoids and tocopherol in ripe than unripe fruit. Furthermore, enzyme activity is preserved under the lower temperatures exerted during freeze-drying, while it is inactivated due to the high temperatures exerted during oven-drying.

The levels of tocopherols and sterols in avocado oil do not increase or decrease dramatically with progressive extraction with SC-CO_2, while chlorophyll and carotenoid levels increase significantly. Sterols and tocopherols are located in cell membranes and will be extracted simultaneously with the triglycerides, decreasing only and at the same rate as the triglyceride content in the plant material decreases. Increasing levels of chlorophyll and carotenoid levels, on the other hand, may be related to their location in chloroplast, chromoplast and idioblast cells which are presumably only ruptured in the latter stages of the extraction process.

The OSI of avocado oil increases with progressive extraction with SC-CO_2. The increased oxidative stability with progressive extraction has a high positive correlation with the chlorophyll and carotenoid contents of the avocado oil, both of which increase with progressive extraction. The antioxidant effect of the carotenoids and chlorophylls is therefore evident. Although the increased OSI and content of chlorophyll and carotenoids can be linked, further work including antioxidant assays testing the radical scavenging (for example DPPH) as well as the prevention of the formation of radicals (for example the β-carotene bleaching method) could shed more light on how these micro-components exert antioxidant activity in the oil.

The OSI of oil extracted from oven-dried avocado fruit is higher than that of oil from freeze-dried fruit. During oven-drying of avocado fruit, chlorophyll may be converted

to pheophytin and pyropheophytin, which are both known to have antioxidant potential and could explain the higher OSI of oil from oven-dried fruit compared to freeze-dried. Although the breakdown products of chlorophyll were not quantified, the AOCS method used to determine chlorophylls is based on the determination of pheophytin equivalents and this indicates the formation of pheophytin in oven-dried fruit. It will however be more meaningful to quantify the breakdown products of chlorophyll with high performance liquid chromatography (HPLC). This will enable the researcher get a clearer picture of how these breakdown products influence the OSI as observed for oil from oven-dried fruit. It is also recommended that tests are performed in future to determine whether phenolic antioxidants are present in the oil as these can also contribute to the antioxidant potential in the oil.

It is also recommended that the metal content of the oil be determined in future studies as compounds such as copper have a strong prooxidant effect in oils and might also contribute to the oxidative stability of the oil.

The OSI of avocado oil extracted with SC-CO_2 can be predicted using the micro-component content of the oil, given that the pre-treatment and extraction methods used in this study are duplicated. The predictive models obtained by using the micro-components as variables show potential for prediction of the OSI of avocado oil for application in the oil industry. The models do not identify chlorophyll and carotenoids together as important variables in predicting the OSI without declaring one of them redundant. However, these two micro-components are identified separately as important positive variables in determining the OSI of the avocado oil, while tocopherols have a small positive effect and sterols, a small negative effect. The models developed in this study can be refined further by using a larger sample size. They can also be refined by firstly quantifying the carotenoids and chlorophylls in the oil and thereafter using these as variables in the development of models predicting the OSI of avocado oil extracted with SC-CO_2.

In this study, due to the fact that lipoxygenase and lipase activity was not determined, one could only speculate about the enzyme activity and the effect on

micro-components and oxidative stability of the oil. The results obtained though, are indicative of higher lipoxygenase and lipase activity in ripe, freeze-dried fruit. However, the determination thereof by enzyme assays and the inclusion of these results in the models will make a significant contribution to the prediction of the shelf-life of avocado oil extracted with SC-CO_2.

Lastly, the SC-CO_2 extraction can be repeated on a laboratory scale plant where the system can be kept at a constant temperature and the flow rate and subsequently volume of CO_2 used to extract the oil can be monitored better. The CO_2 volume required to extract a certain amount of oil, can in return, be correlated with the levels of micro-components in the respective oil samples.

This study demonstrates that avocado oil can be enriched in micro-components through progressive extraction with SC-CO_2. This has important consequences for the oxidative stability. The pre-treatment (fruit ripeness and drying) is an important factor to consider as it affects the yield as well as the chemical composition of the oil and hence the oxidative stability. The initial capital investment as well as running costs to operate drying facilities and the SC-CO_2 extraction unit should also be considered.

The cost, yield and quality of avocado oil produced with conventional methods should be compared to that of avocado oil extracted with SC-CO_2. SC-CO_2 may be a useful tool in the quest to diversify the application of avocado oil by producing oil enriched with micro-components which may be oxidatively more stable and can provide potential health benefits.

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6. PUBLICATIONS AND POSTERS

Publication:

Mostert, M.E., Botha, B.M., Du Plessis, L.M. & Duodu, K.G., 2007. Effect of fruit ripeness and method of fruit drying on the extractability of avocado oil with hexane and supercritical carbon dioxide. *Journal of the Science of Food and Agriculture*, **87**, 2880-2885. DOI: 10.1002/jsfa.3051

Poster:

Mostert, M.E., Botha, B.M., Du Plessis, L.M. & Duodu, K.G., 2007. Oxidative stability of avocado oil extracted with supercritical carbon dioxide: Effect of fruit pre-treatment, progressive extraction and micro-components. CHEMRAWN XII Conference on The Role of Chemistry in Sustainable Agriculture and Human Wellbeing in Africa, Stellenbosch University, South Africa.

Oral papers:

Mostert, M.E., Botha, B.M., Du Plessis, L.M. & Duodu, K.G., 2005. The Supercritical Carbon Dioxide Extraction and Oxidative Stability of Avocado Oil. South African Association for Food Science & Technology (SAAFoST) 18th Biennial Conference, Stellenbosch, South Africa.

Mostert, M.E., Botha, B.M., Du Plessis, L.M. & Duodu, K.G., 2007. Effect Of Fruit Pre-Treatment And Progressive Extraction On Oxidative Stability Of Avocado Oil Extracted With Supercritical Carbon Dioxide. South African Association for Food Science & Technology (SAAFoST) 19th Biennial Conference, Durban, South Africa.

Mostert, M.E., Botha, B.M., Du Plessis, L.M. & Duodu, K.G., 2007. Characterization of micro-components of avocado oil extracted with supercritical carbon dioxide and their effect on its oxidative stability. European Federation of Lipid Science, 5th Congress, Gothenburg, Sweden.