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).

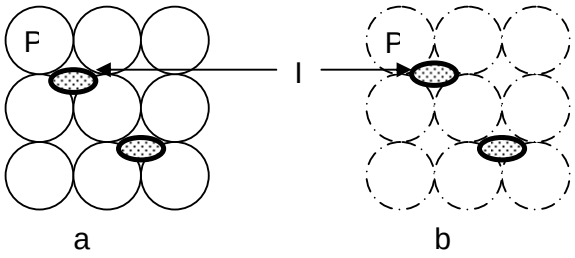


Figure 3.1: Sketch illustrating unripe avocado mesocarp cells where the cell walls of the parenchyma cells (P) are still intact (a), and ripe avocado mesocarp (b) where the parenchyma cell walls have been damaged by cell wall degrading enzymes. The idioblast cells (I) with their thickened cell walls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

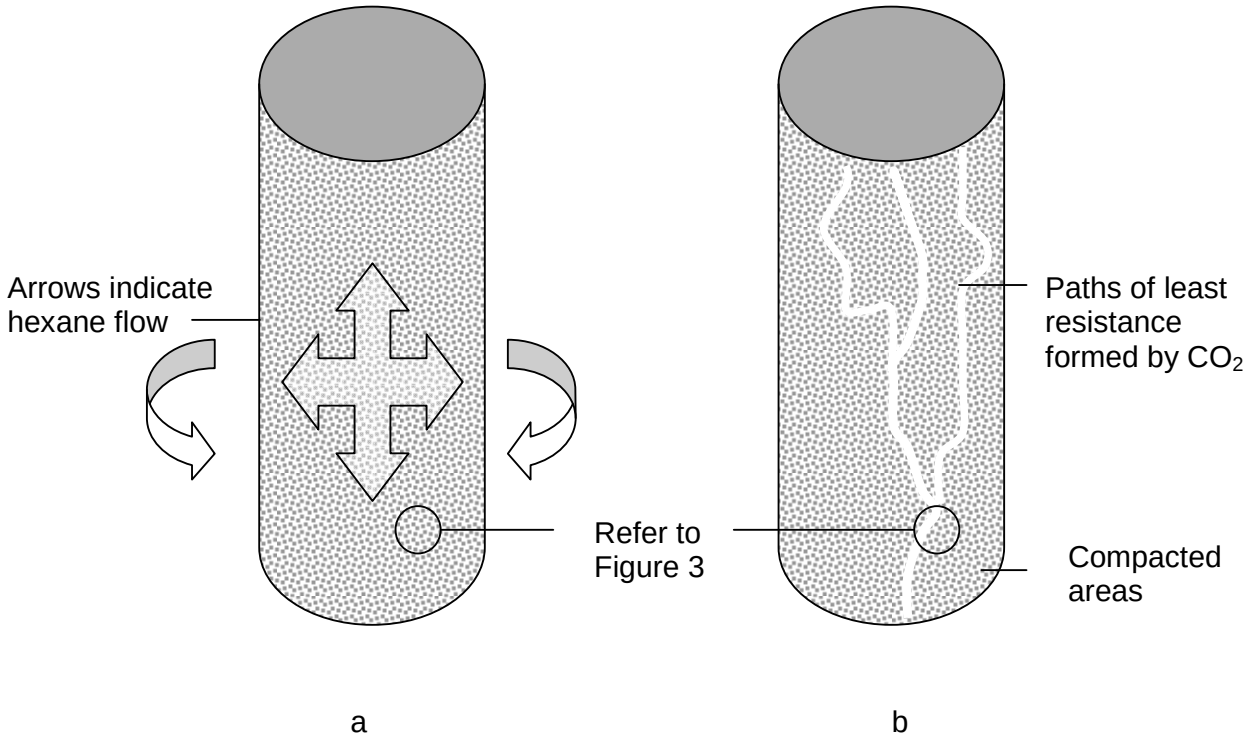


Figure 3.2: Figures illustrating the movement of hexane (a), and supercritical carbon dioxide (SC-CO_2) (b) through the plant material. Hexane flows through and around all particles because the particles are immersed in and boiled in the solvent. SC-CO_2 is forced through with pressure and paths of least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

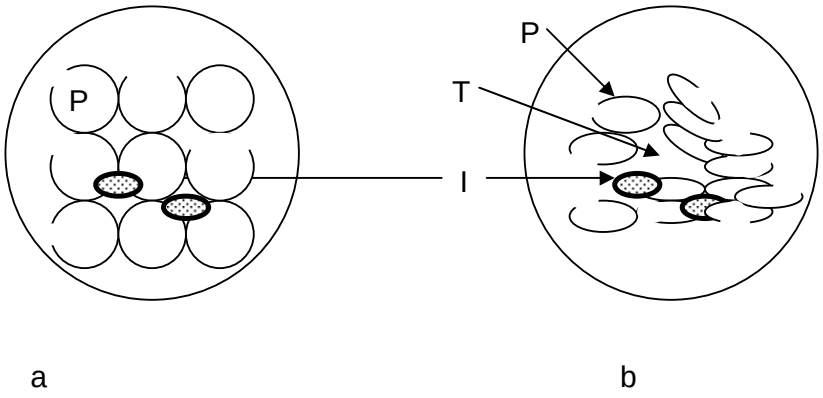


Figure 3.3: Sketch illustrating avocado mesocarp cells after extraction with hexane (a) and after extraction with SC-CO_2 (b) where the cells have been compacted due to the pressure in the cylinder and "tunnels" have been formed where the SC-CO_2 followed the path of least resistance. P = parenchyma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

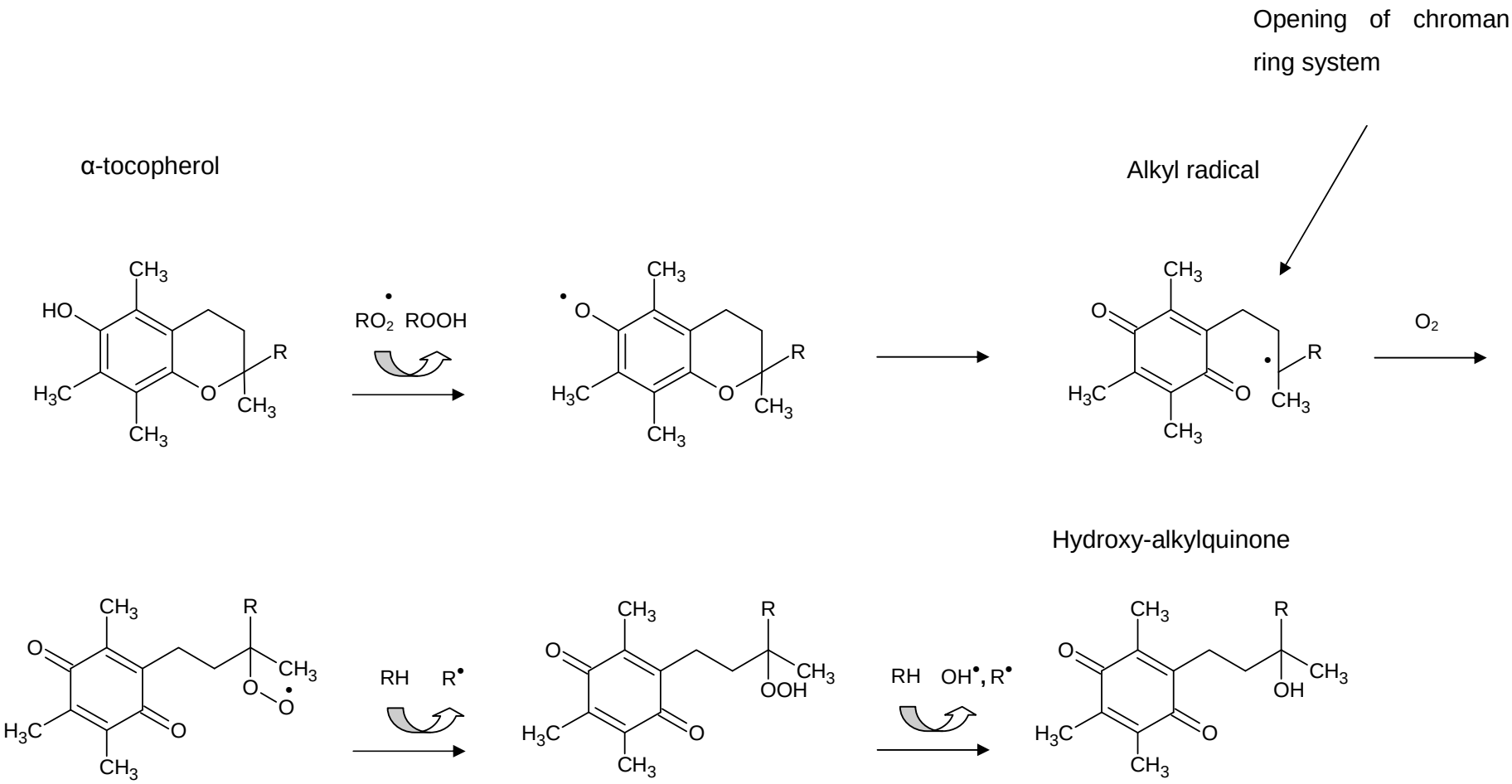


Figure 3.4: Schematic illustration of the oxidative deterioration of α-tocopherol (Belitz *et al*., 2004[a])

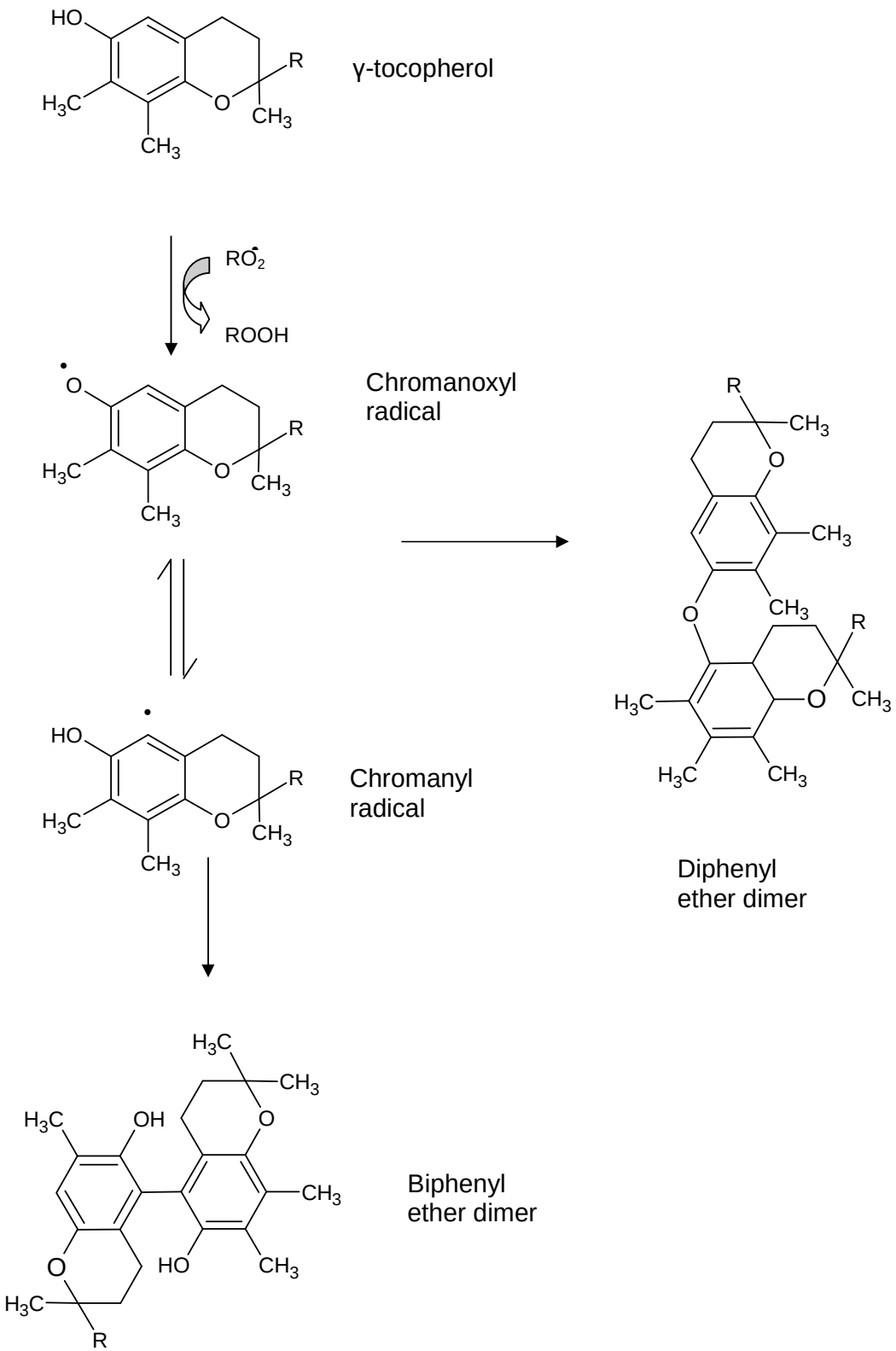


Figure 3.5: Schematic illustration of the oxidative deterioration of γ-tocopherol (Belitz *et al*., 2004[a])

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]).

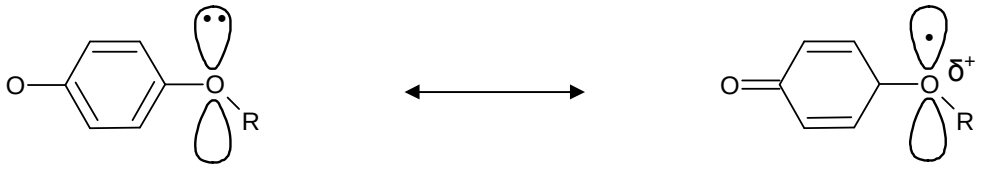


Figure 3.6: Stabilising of the chromanoxyl radical by resonance

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.

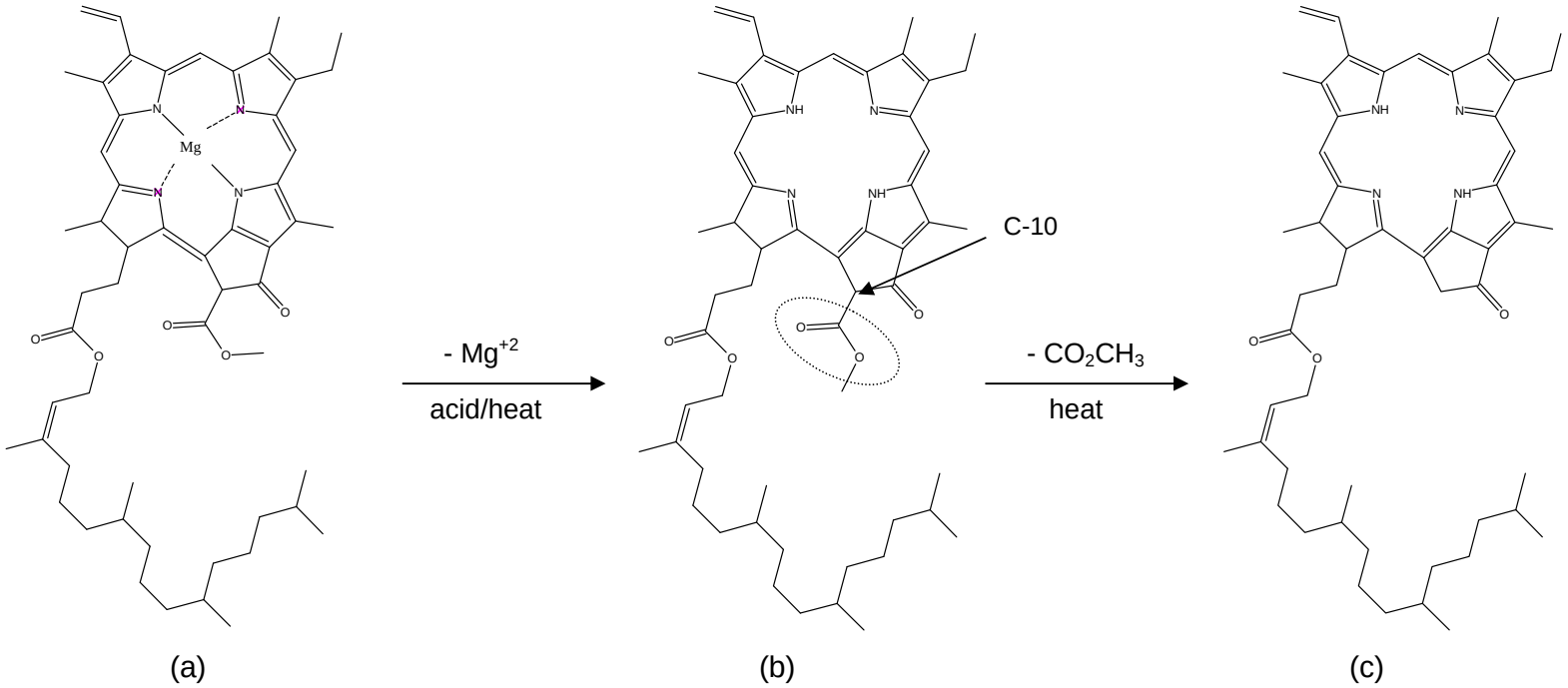


Figure 3.7: The thermal degradation process of (a) chlorophyll *a* to (b) pheophytin *a* and (c) pyropheophytin *a*.

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.

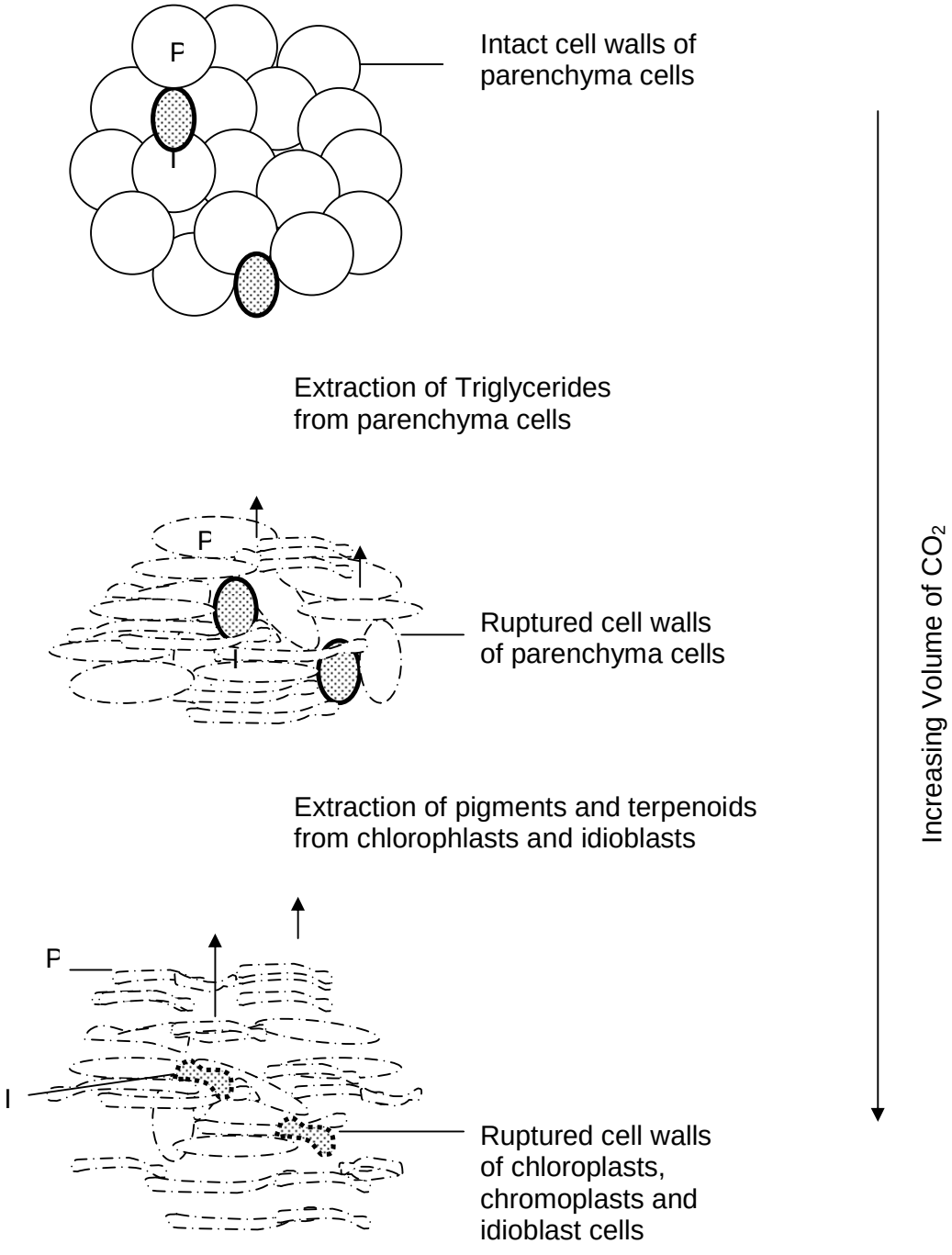


Figure 3.8: Figures illustrating the destruction of cellular integrity during progressive extraction with SC-CO_2 indicating how triglycerides were probably extracted first, followed by pigments and terpenoids contained within more rigid structures.

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).

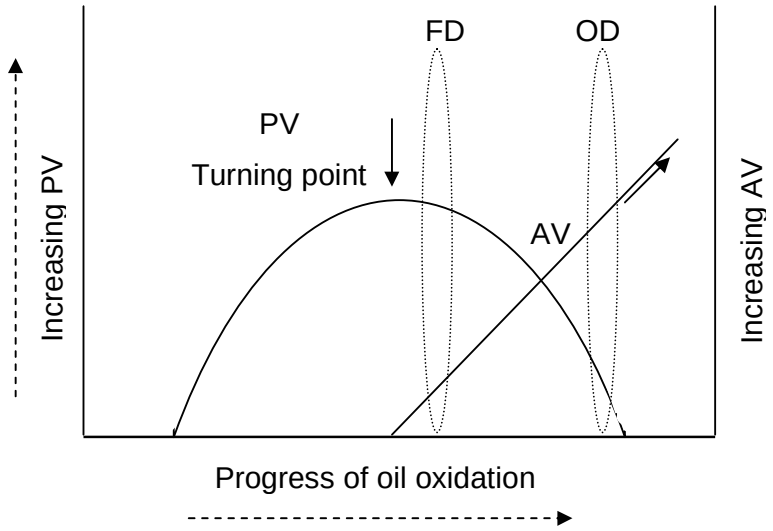


Figure 3.9: Typical peroxide value (PV) and anisidine value (AV) curves depicting the different oxidation rates of oil extracted from freeze-dried (FD) and oven-dried (OD) fruit.

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.

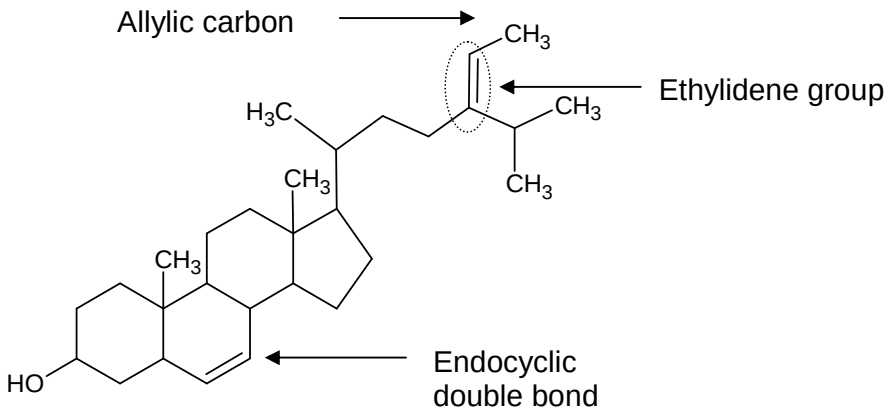
Although the antioxidant effects of tocopherols are well-known (Murcia *et al*., 2001), the only significant correlation between the OSI value and any of the tocopherol isomers, including total tocopherol, was for δ-tocopherol (-0.60) (the most abundant isomer in avocado oil) indicating a pro-oxidant 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.

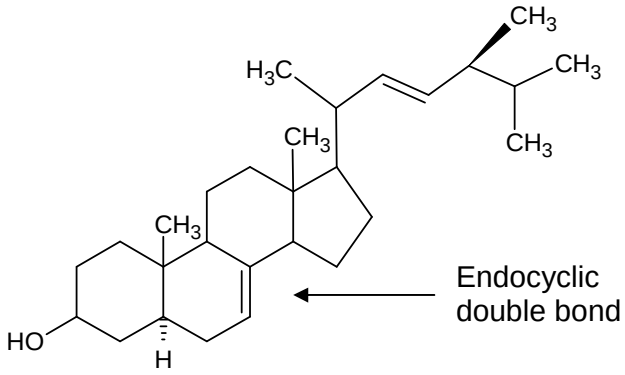
The stabilizing effect of some sterols in oil subjected to prolonged heating have been reported by various authors (Sims *et al*. 1972; Boskou & Morton, 1976; White & Armstrong, 1986). A minor, non-significant positive effect of total sterols on the OSI of extra virgin olive oil have been reported by 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.