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]).

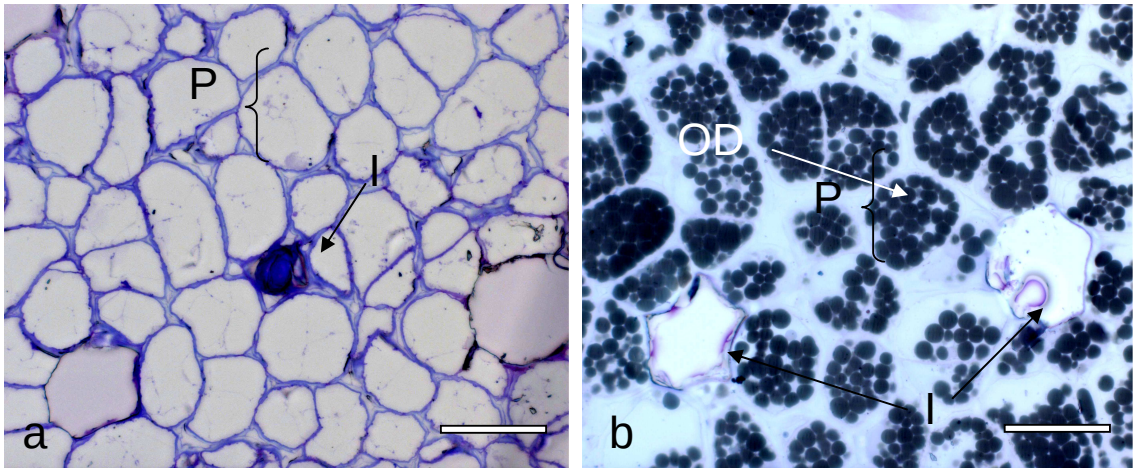

Figure 2.1.1: Avocado mesocarp stained with toluidene blue alone (a) and Avocado mesocarp stained with toluidene blue and fixed with osmium tetroxide. I: Idioblast cells; OD: Oil droplets; P: Parenchyma cell. Bar represents 10 µm.

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.

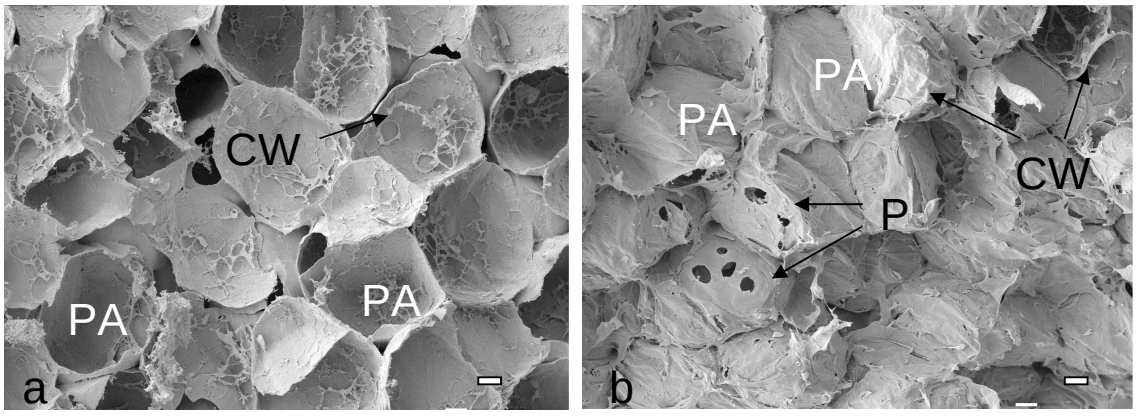

Figure 2.1.2: Scanning electron micrographs of fresh unripe (a) and ripe (b) avocado mesocarp showing parenchyma cells (PA), cell walls (CW) and pits (P). Bar represents 10 µm.

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.

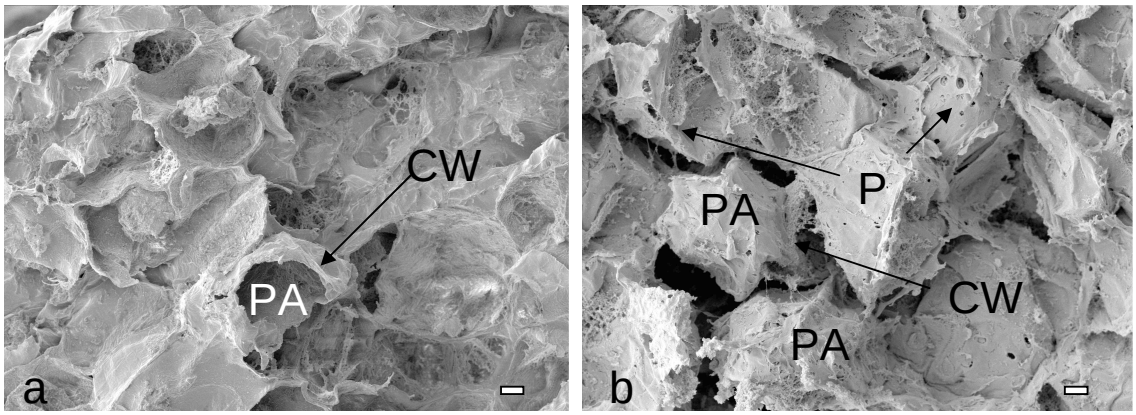

Figure 2.1.3: Scanning electron micrographs of unripe (a) and ripe (b) avocado mesocarp after deep freezing at -20℃ showing parenchyma cells (PA), cell walls (CW) and pits (P). Note partial separation of parenchyma cells in ripe fruit sample (b). Bar represents 10 µm.

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.

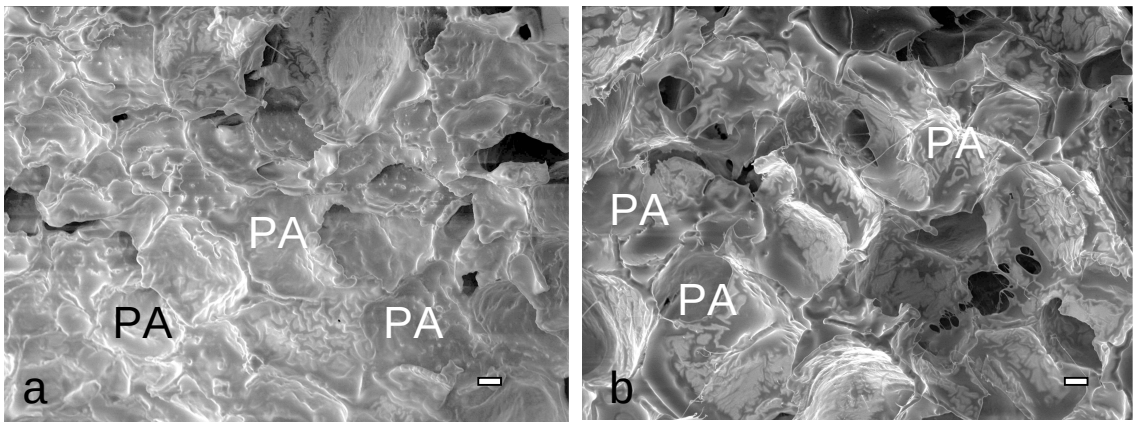

Figure 2.1.4: Scanning electron micrographs of unripe (a) and ripe (b) avocado mesocarp after freeze-drying showing irregularly shaped and flattened parenchyma cells (PA). Bar represents 10 µm.

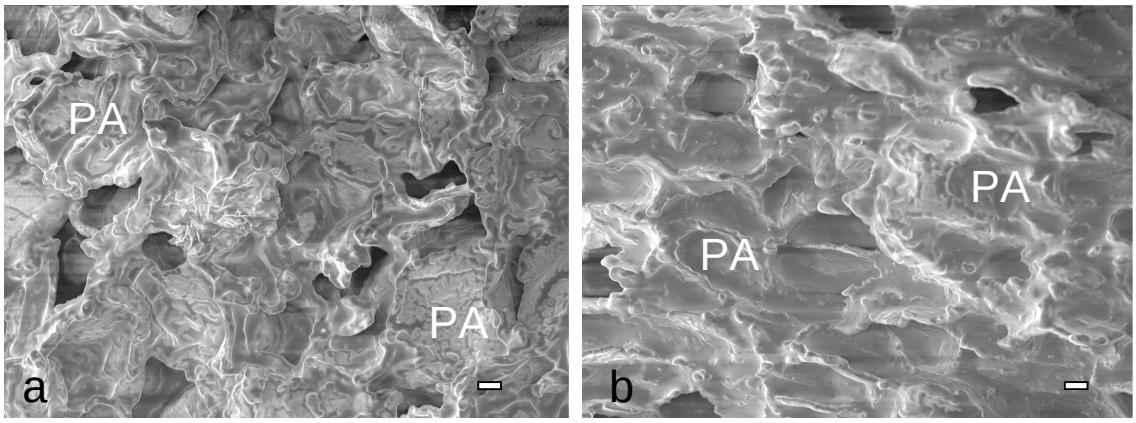

Figure 2.1.5: Scanning electron micrographs of unripe (a) and ripe (b) avocado mesocarp after oven-drying at 80℃ showing irregula rly shaped and flattened parenchyma cells (PA). Bar represents 10 µm.

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.

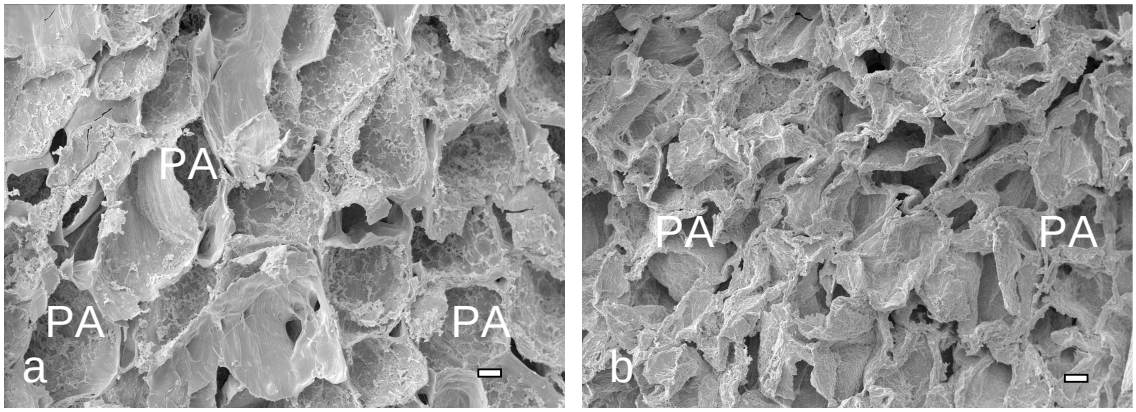

Figure 2.1.6 Scanning electron micrographs of unripe freeze-dried (a) and unripe oven-dried (b) avocado mesocarp after hexane extraction. Note the presence of empty parenchyma cells (PA) throughout the plant material. Bar represents 10 µm.

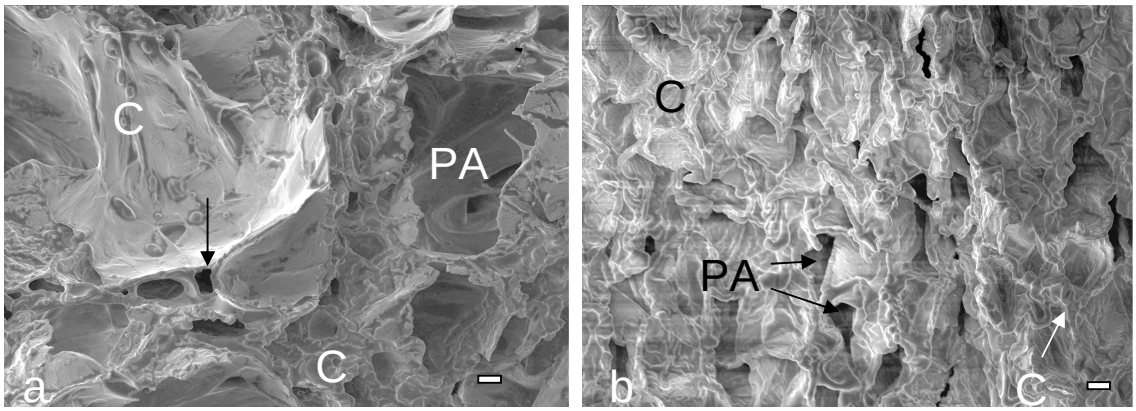

Figure 2.1.7: Scanning electron micrographs of unripe freeze-dried (a) and unripe oven-dried (b) avocado mesocarp after supercritical carbon dioxide (SC-CO_2) extraction. Note compacted areas (C) and loose areas with empty parenchyma cells (PA). Bar represents 10 µm.

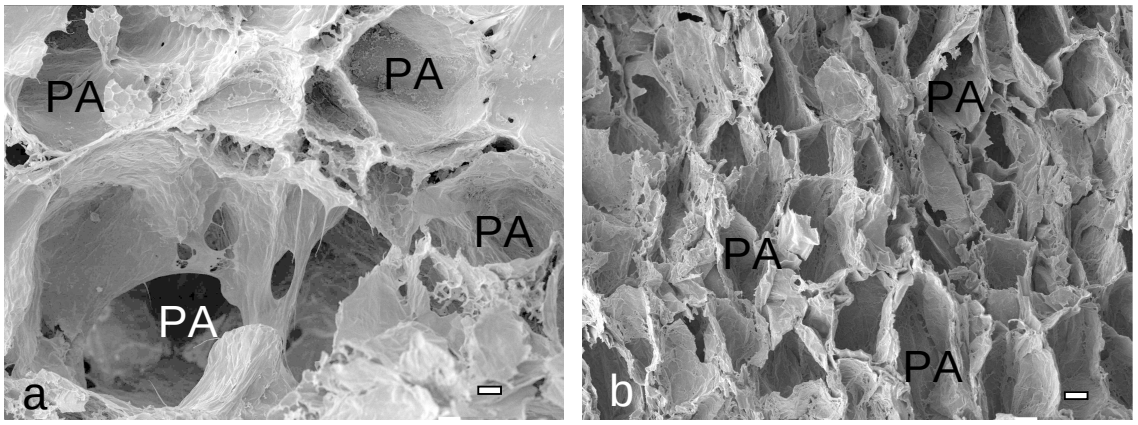

Figure 2.1.8: Scanning electron micrographs of ripe freeze-dried (a) and ripe oven-dried (b) avocado mesocarp after hexane extraction. PA – empty parenchyma cells. Bar represents 10 µm.

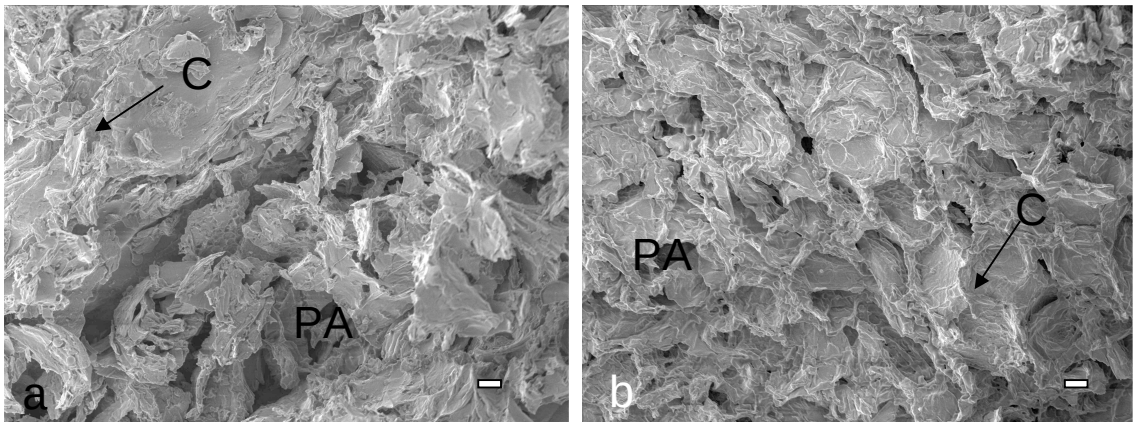

Figure 2.1.9: Scanning electron micrographs of ripe freeze-dried (a) and ripe oven-dried (b) avocado mesocarp after supercritical carbon dioxide (SC-CO_2) extraction. C – compacted areas; PA – empty parenchyma cells in loose areas. Bar represents 10 µm.

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

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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.

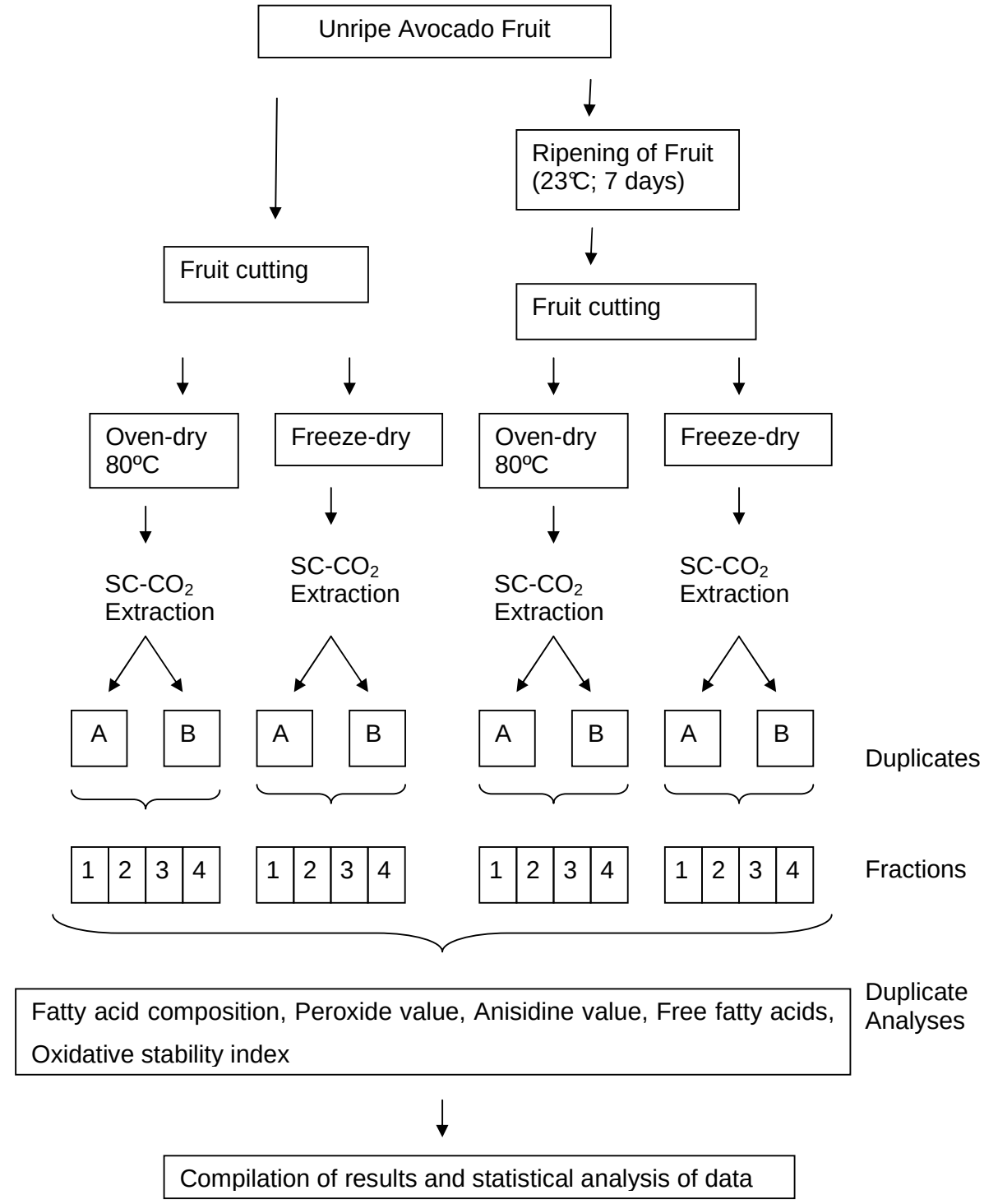

Figure 2.2.1: Summary of experimental design for avocado fruit pre-treatment, avocado oil extraction and oxidative stability tests.

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.

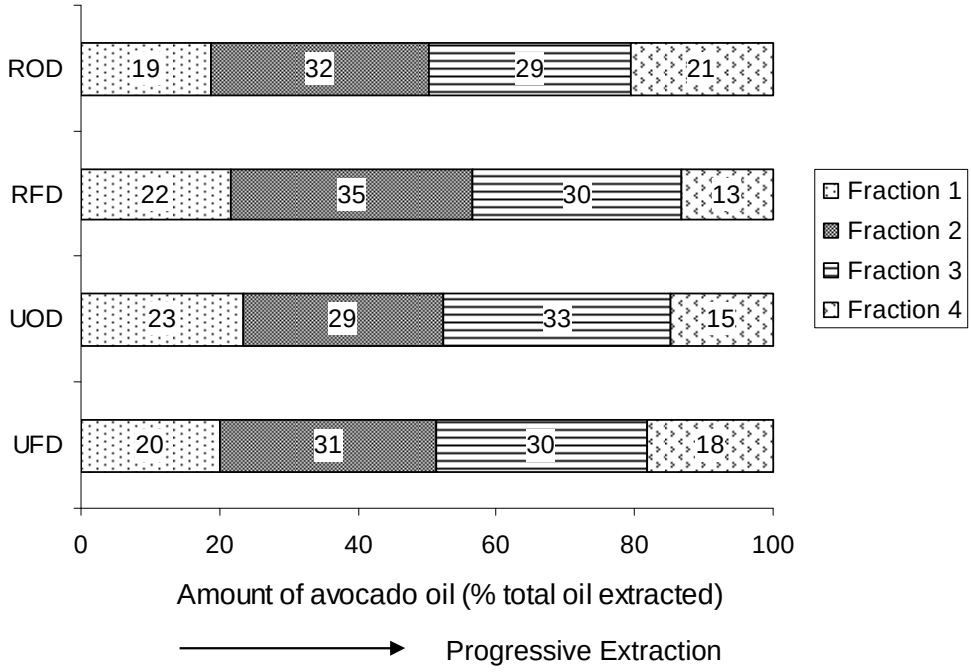

Figure 2.2.2: Division of avocado oil into four consecutive fractions during progressive extraction with supercritical carbon dioxide (SC-CO_2).

* 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 $p < 0.05$.

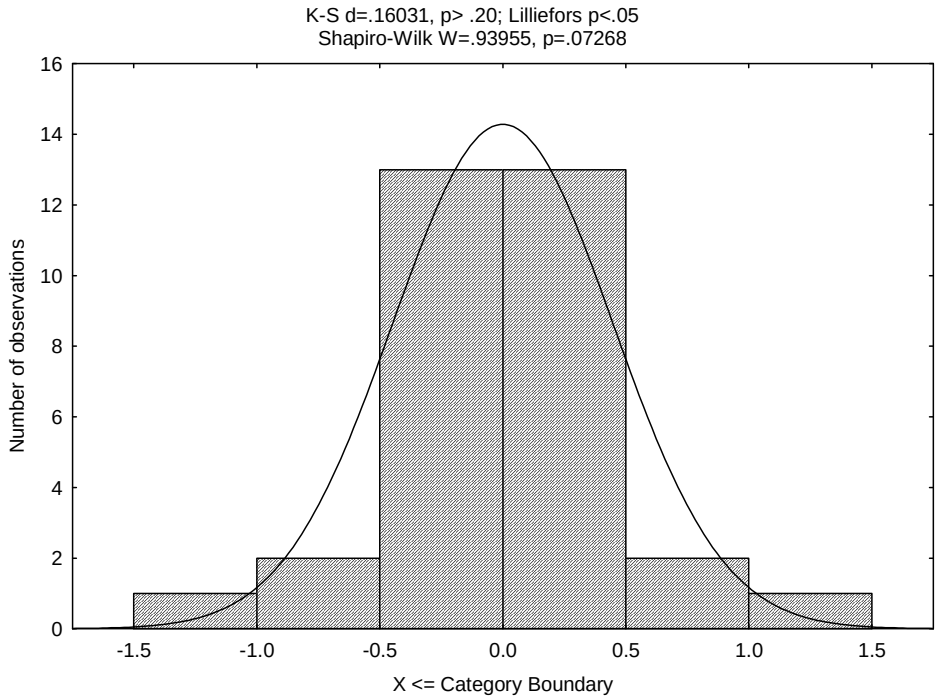

Figure 2.2.3 Histogram displaying the normality of the data as observed from plotting the residuals for all the values obtained for the Anisidine value. Results for the test for normality were as follows: K-S d = 0.16031, $p > 0.20$; Lilliefors $p > 0.05$; Shapiro-Wilk W = 0.93955, p = 0.7268. This histogram 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.

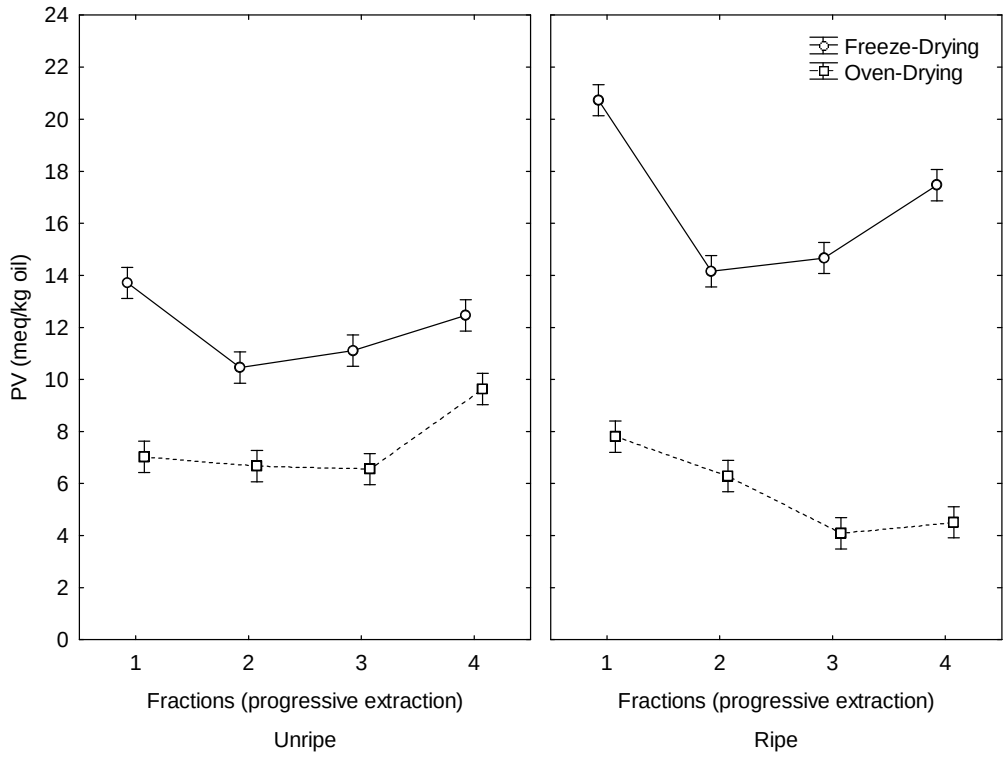

Figure 2.2.4: Peroxide value of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

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).

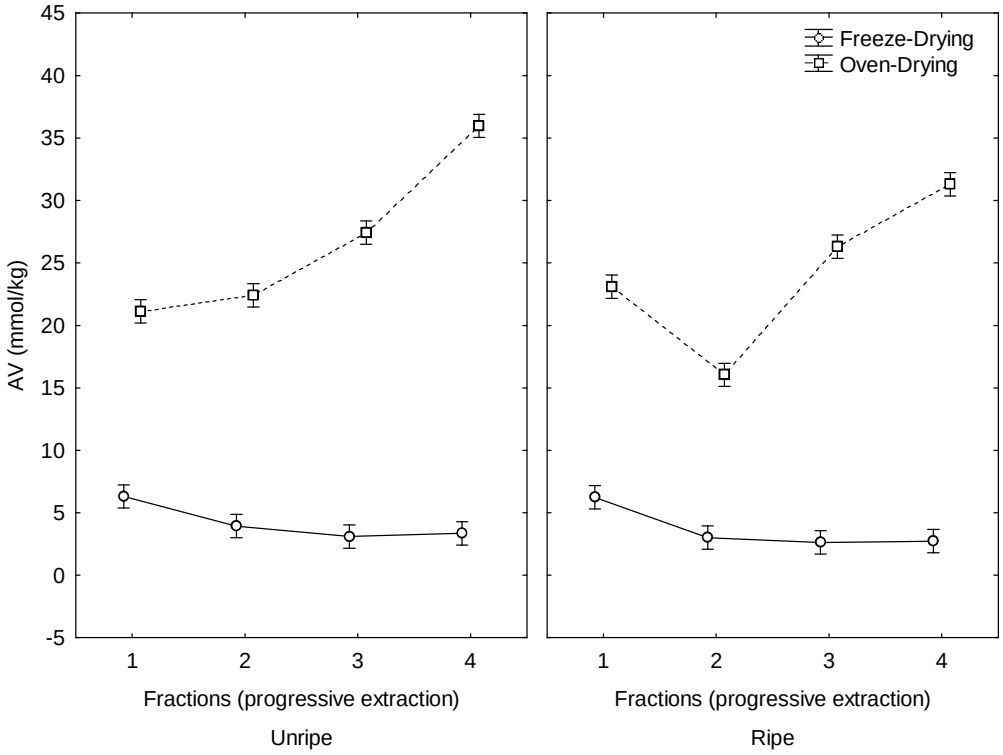

Figure 2.2.5: Anisidine value of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

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.

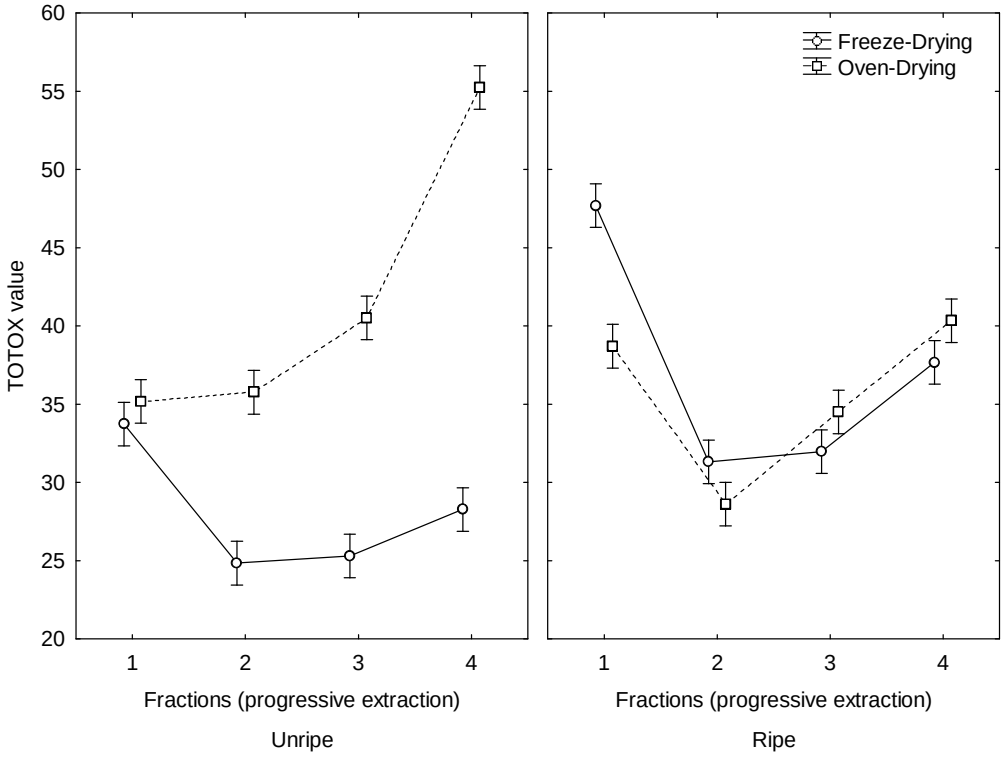

Figure 2.2.6: TOTOX value of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

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.

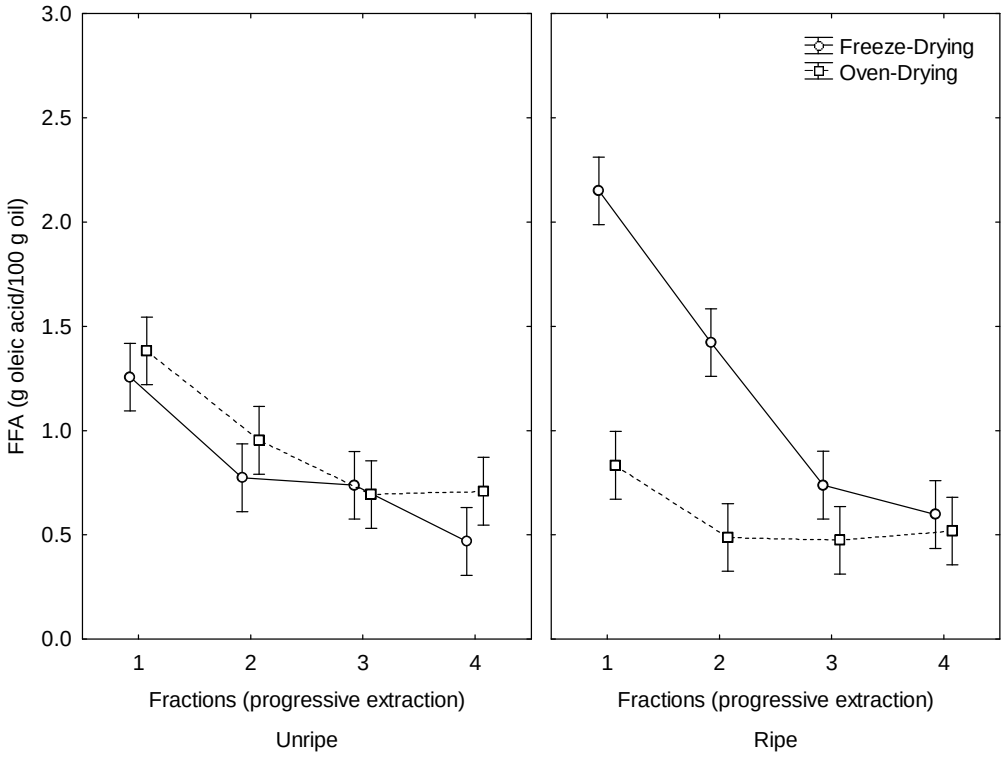

Figure 2.2.7: Free fatty acid content of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2) Vertical bars denote 0.95 confidence intervals.

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*).

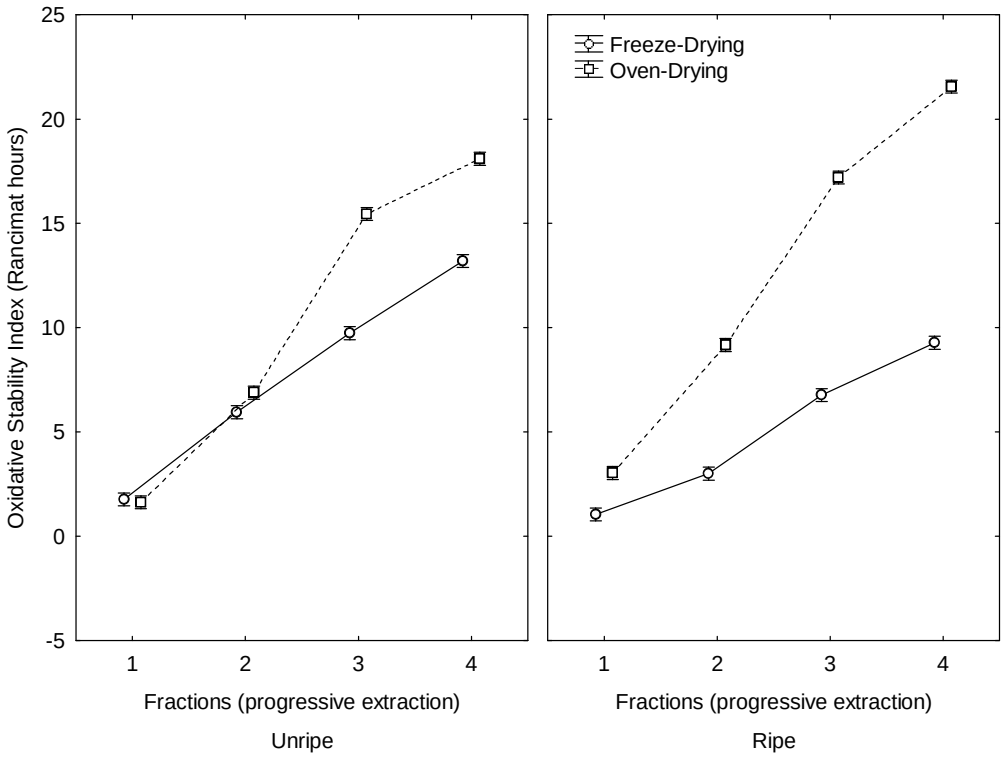

Figure 2.2.8: Oxidative stability index (Rancimat hours) of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

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.

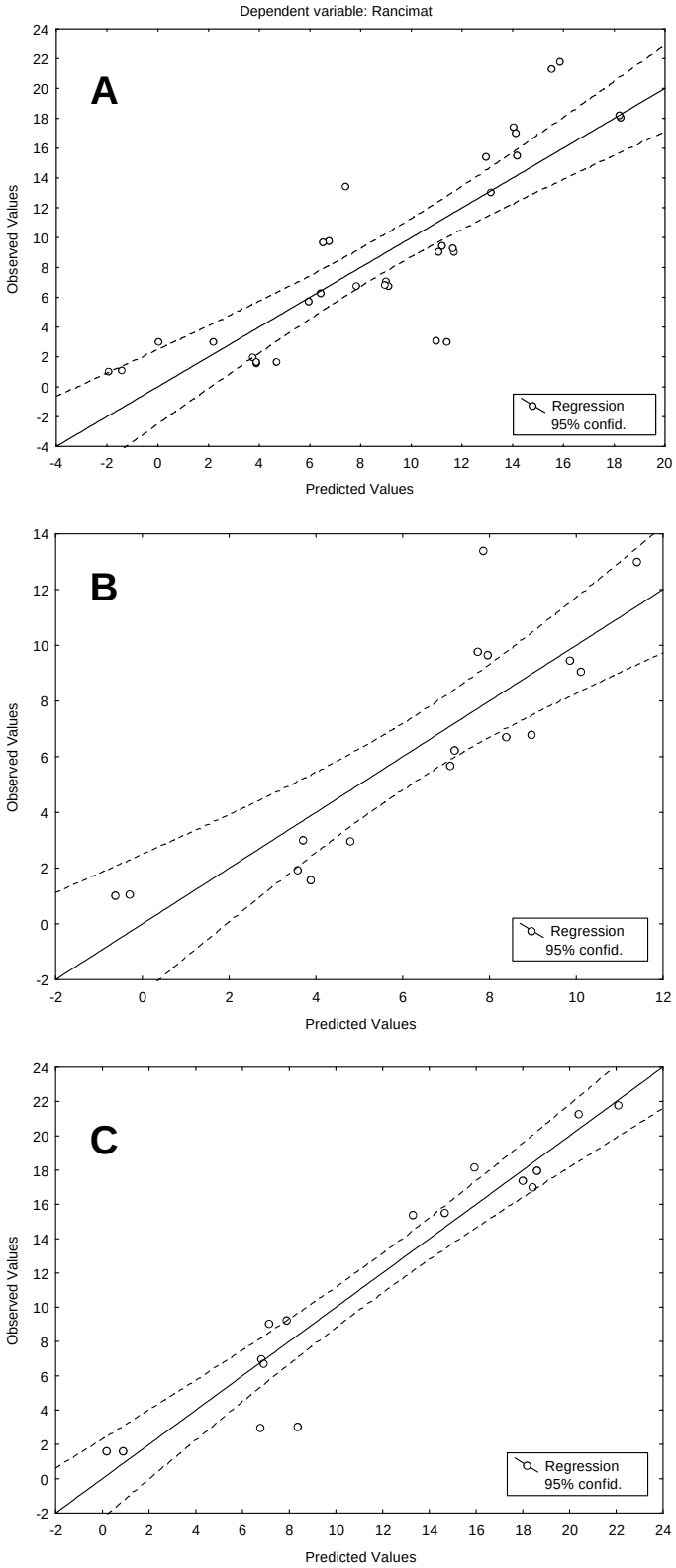

Figure 2.2.9: Predicted versus observed oxidative stability index values for avocado oil extracted with supercritical carbon dioxide (SC-CO_2). A – oil from freeze-dried and oven-dried fruit; B – oil from freeze-dried fruit; C – oil from oven-dried fruit.

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.

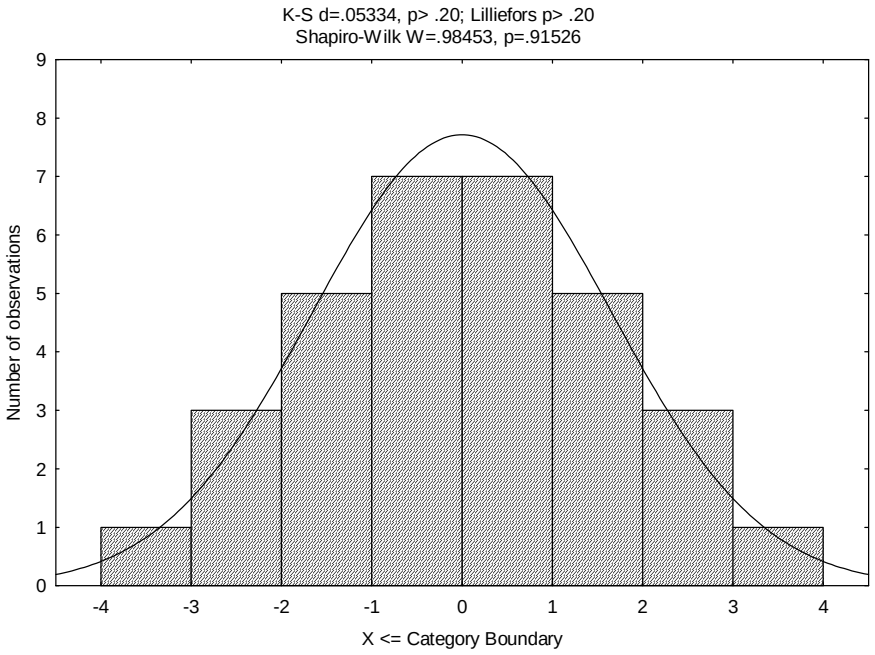

Figure 2.3.1: Histogram displaying the normality of the data as observed from plotting the residuals for all the values obtained for campesterol. Results for the test for normality were as follows: K-S d = 0.05334, p > 0.20; Lilliefors p > 0.20; Shapiro-Wilk W = 0.98453, p = 0.91526. This histogram is 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.

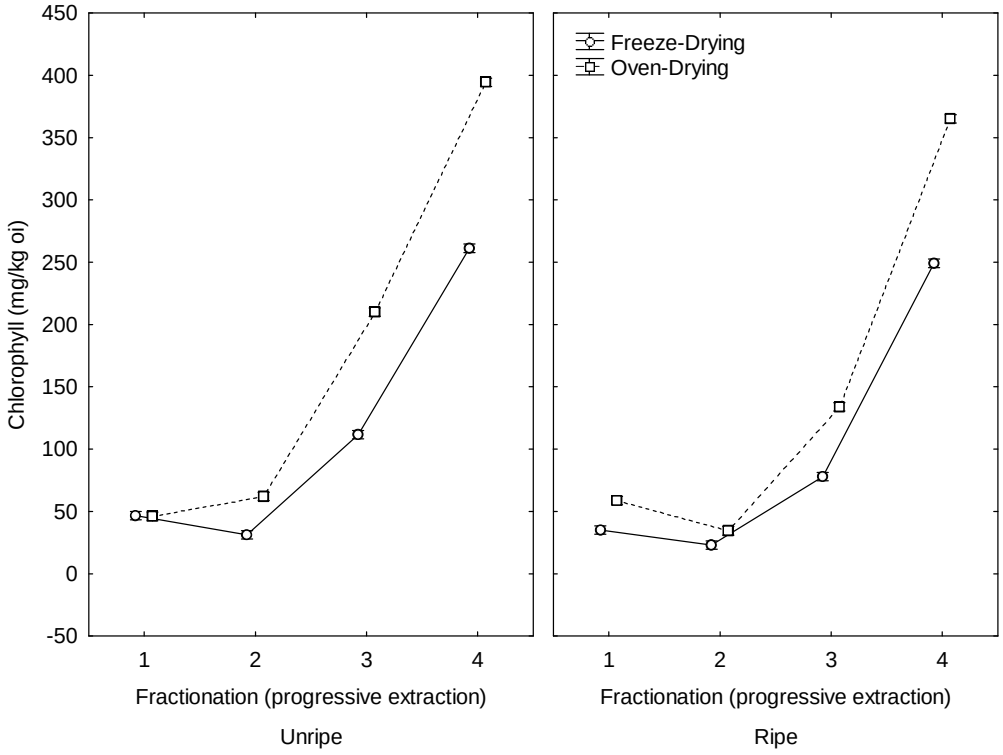

Figure 2.3.2: Chlorophyll content of oil (mg pheophytin/kg oil) extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

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).

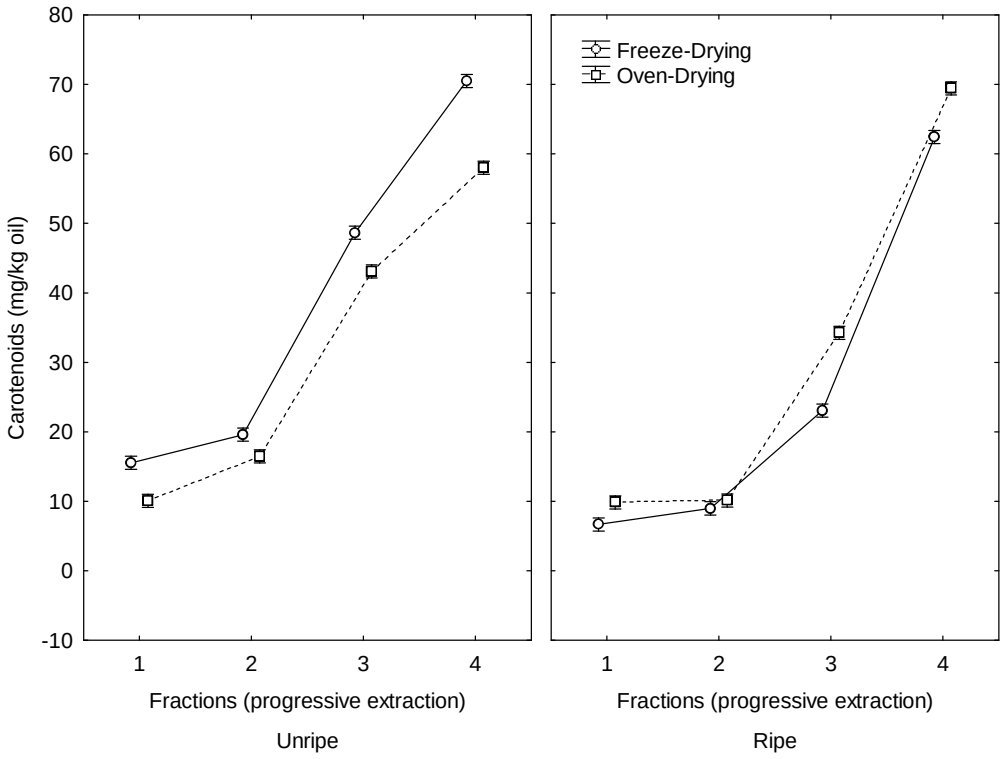

Figure 2.3.4: Carotenoid content (mg/kg oil) of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

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).

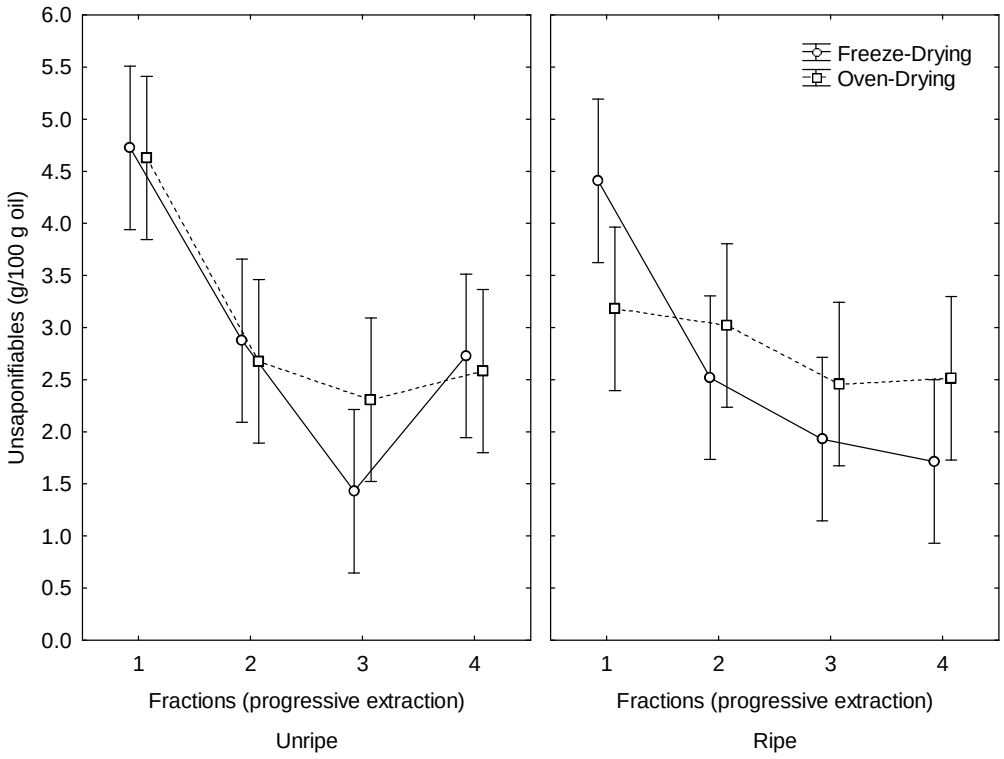

Figure 2.3.5: Unsaponifiable content (g/100 g oil) of oil extracted from avocado fruit with supercritical carbon dioxide (SC-CO_2). Vertical bars denote 0.95 confidence intervals.

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.

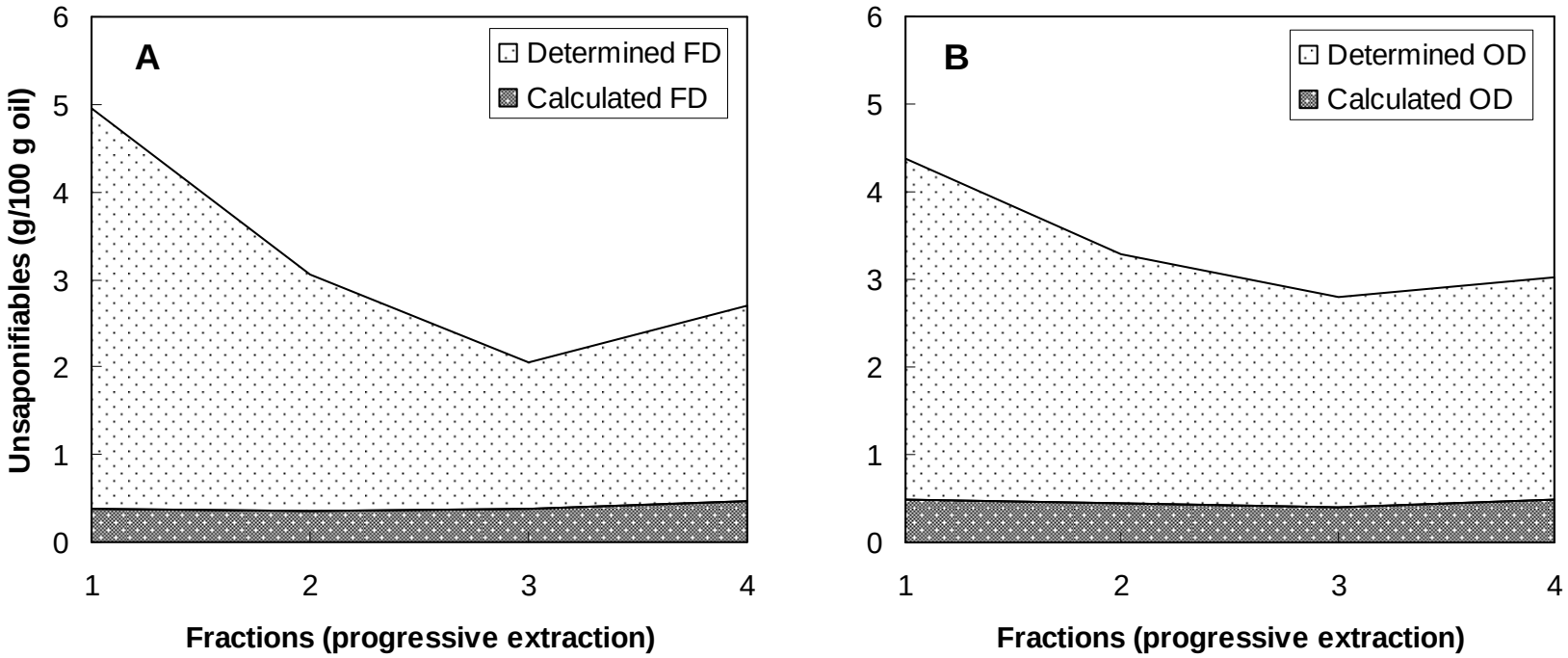

Figure 2.3.6: Unsaponifiable values determined with AOCS method Ca 6a-40 and unsaponifiable values calculated by adding the micro-components (carotenoids, chlorophyll, tocopherols and sterols) together for oil extracted from freeze-dried (a) and oven-dried (b) avocado fruit with supercritical carbon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

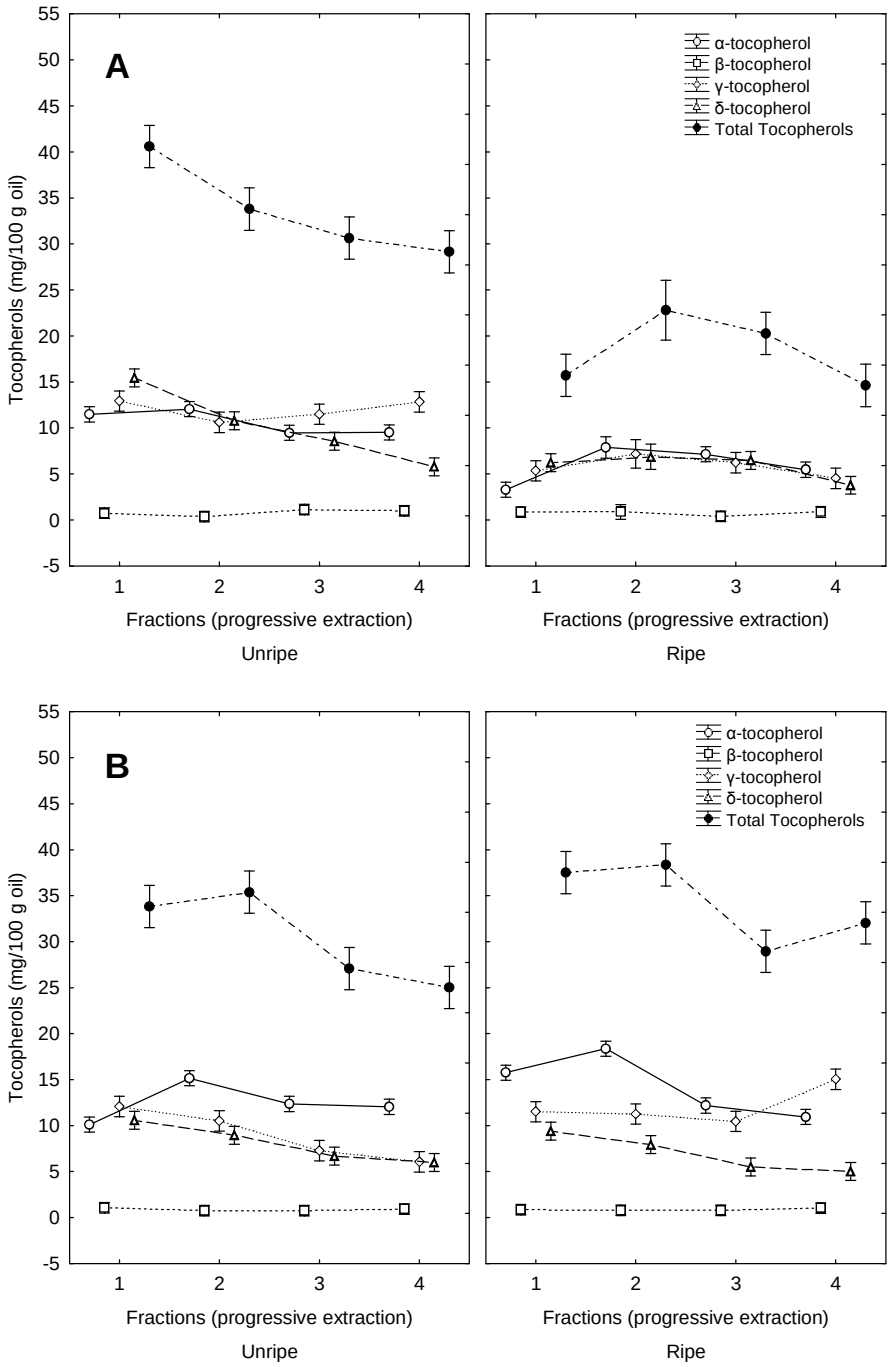

Figure 2.3.7: Tocopherol content of oil extracted from (A) freeze-dried and (B) oven-dried avocado fruit with supercritical carbon dioxide (SC-CO_2). (Total = alpha + beta + gamma + delta). Vertical bars denote 0.95 confidence intervals.

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.

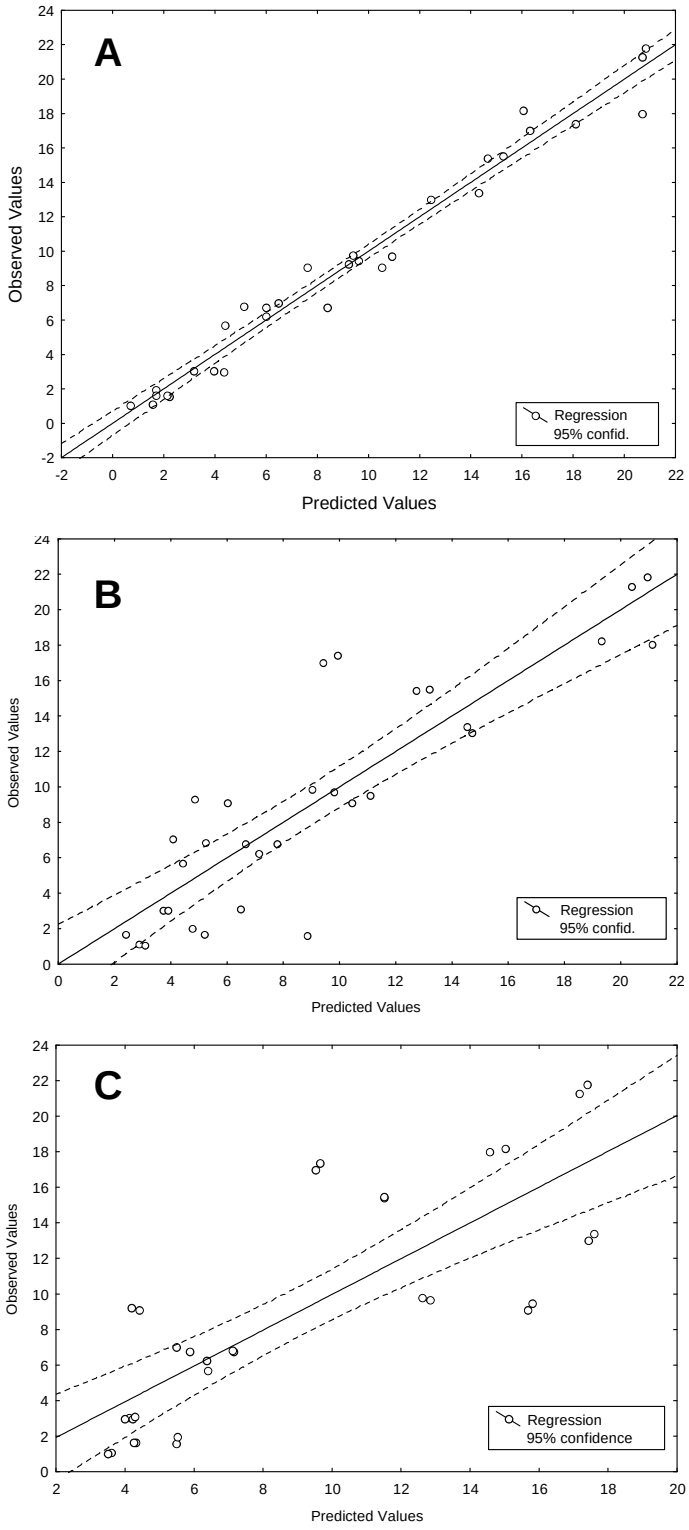

Figure 2.3.8: Predicted versus observed oxidative stability index values based on micro-component content for avocado oil extracted with supercritical carbon dioxide (SC-CO_2) for the three models.

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

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