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The Oxidation Stability of Extra Virgin Avocado Oil

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Abstract

Extra virgin avocado oil (EVAO) is extracted from avocado fruit with minimal processing. It contains a wide range of non-lipid compounds that have a profound affect on oil stability. The deterioration of oil quality is due to autoxidation and photooxidation reactions that occur during oil storage. The objectives of this research were to determine the effect of prooxidant factors (light, temperature, oxygen level) on oil oxidation and quality; make recommendations for oil processing and packaging procedures to minimise oxidation; predict the shelf life of the oil and to determine the effect of commercial antioxidants on oil oxidation.

An accelerated oxidation reactor was developed to test the effects of fluorescent light, elevated temperature and varying oxygen levels on the peroxide value (PV) (initially 0.96 ± 0.03 meq/kg oil) and chlorophyll content (initially 16.2 ± 0.1 ppm) of EVAO. The production and packaging processes of Olivado NZ were analysed for exposure to oxidation promoting factors. EVAO was exposed to dark storage at 50°C and 60°C in order to determine Q_{10} values for oil oxidation. Several commercial antioxidants were evaluated by examining their affect on EVAO using the Rancimat oil stability index analysis and hot air oven testing.

It was found that fluorescent light at 4500 lux and aeration with dry air strongly accelerated the oxidation (determined by PV) and reduced the chlorophyll content of EVAO. The average effect of 4500 lux fluorescent light compared to 0 lux over seven hours was a PV increase of 4.5 ± 1.4 meq/kg oil and decrease in chlorophyll content by 0.9 ± 0.3 ppm. The average effect of aerated EVAO compared to EVAO stored at ambient oxygen levels over seven hours was a PV increase of 3.5 ± 1.7 meq/kg oil and a chlorophyll content decrease of 0.3 ± 0.2 ppm. Exposure to an elevated temperature of 60°C for seven hours did not cause a significant increase in PV. Recommendations were made to minimise the exposure of the oil to light, aeration, water and fruit sediment during production and packaging in order to minimise oxidation of the oil. Due to the breakdown of natural antioxidants and alternative side reactions that occurred at elevated test temperatures but not at ambient temperatures, the shelf life of the oil could not be defined. EVAO containing ascorbyl palmitate at a level of 100 ppm had a peroxide value 80 % less than control EVAO with no antioxidants after 500 hours storage at 60°C. Ascorbyl palmitate has GRAS status and was concluded to be the most effective antioxidant of those tested in EVAO.

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1 Introduction

Avocado production in New Zealand increased from 1000 tonnes in 1987 to 3250 tonnes in 1997 (Requejo-Tapia, 1999). A 15% growth in avocado production each year since 1995 and an almost an almost two-fold increase in the area under cultivation for this crop suggests that there will be a substantial increase in avocado production in the future, as shown in Figure 1.

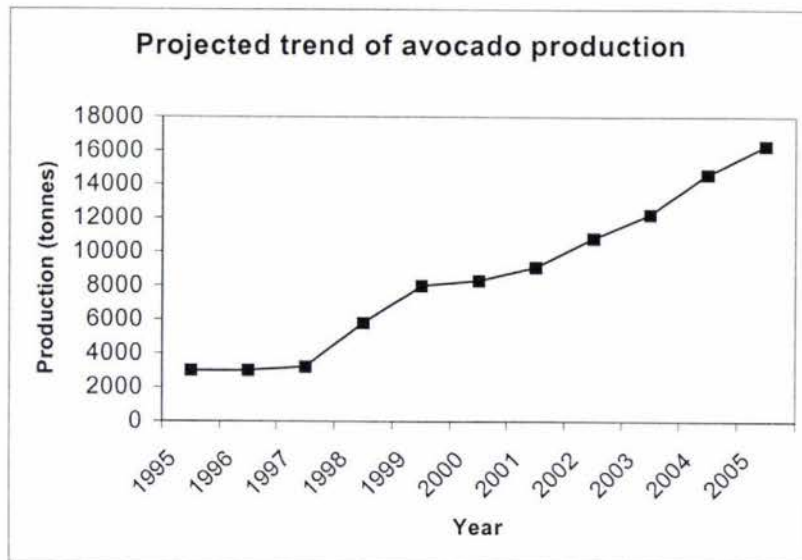


Figure 1. Projected trend of avocado production in New Zealand (Requejo-Tapia, 1999)

The large increase in avocado production also suggests a large increase in fruit that will not meet export quality criteria. The small population of New Zealand can only consume a limited amount of these second grade fruit and over-supply is likely to result in a drop in avocado prices, making them less economical for growers (Eyres et al., 2001).

An alternative to wasting second grade fruit is to turn them into useful by-products. It is well known that avocados have a relatively high oil content, which is comparable to

olives (Werman and Neeman, 1987; Kiritsakis, 1998). This oil can be extracted as a way of utilising surplus fruit as well as producing a value added product (Human, 1987). Currently, there are two processing plants in New Zealand that can produce high quality, extra virgin avocado oil (EVAO).

Edible oils consist mostly of triacylglyceride molecules (Hamilton, 1994). These are made up of a glyceride backbone with three fatty acids attached (Lawson, 1995). The composition of these fatty acids in the oil strongly affects the nutritional value, physical state and stability of the oil (Lawson, 1995). Traditional vegetable oils, such as soybean oil, consist of mostly polyunsaturated fatty acids, fatty acids with more than one carbon-carbon double bond each. They have a neutral affect on arterial health but their high degree of unsaturation makes them very susceptible to oxidative degradation (Baur, 1995; Nawar, 1996). Animal fats such as lard and milk fat consist of mostly saturated fatty acids, fatty acids with no carbon-carbon double bonds. Saturated fats tend to increase blood cholesterol levels and the associated risk of cardiovascular disease and strokes (Baur, 1995). The saturated bonds of the fatty acids are however very stable to oxidation (Nawar, 1996). Other types of vegetable oils including canola oil, olive oil and avocado oil are very high in monounsaturated fatty acids, fatty acids with only one carbon-carbon double bond (Kiritsakis, 1998; Eyres et al., 2001). This type of fatty acid has a beneficial affect on arterial health, and consumption can lower blood cholesterol (WHO, 1982; Baur, 1995). Monounsaturated fatty acids are also very stable to oxidative degradation (Kiritsakis, 1998). A large body of scientific evidence supports a move from the typical Western diet, rich in saturated fat, to a more healthy diet similar to that consumed in the Mediterranean area, rich in monounsaturated oils (Birkbeck, 2002).

Extra virgin olive oil and avocado oil are produced using minimal processing at low temperatures. These oils retain many of the characteristics of the fruit from which they were extracted, including natural antioxidants, unique flavours and pigments (Rhamani and Csallany, 1998). In contrast, most other edible fats and oils undergo a refining process to produce bland, colourless and odourless oils that do not retain any character of the material from which they were extracted (Lawson, 1995). Avocado oil has an added benefit in that it contains β-sitosterol in significant amounts (0.5-1.0%) and the

consumption of this cholesterol lowering sterol is being encouraged worldwide (Eyres et al., 2001).

Oxidation is a series of chemical reactions that leads to the breakdown of oil and the production of breakdown products that have an undesirable 'rancid' flavour and odour (Hamilton, 1994). It constitutes the main form of deterioration in edible fats and oils during storage (Kiritsakis, 1998). It is generally agreed that 'autoxidation', the reaction with molecular oxygen via a self-catalytic mechanism, is the main reaction involved in the oxidative deterioration of lipids (Nawar, 1996). Oxidation causes off-flavour and odour formation as well as loss in colour and a reduction in vitamin A, vitamin E and essential fatty acids (Nawar, 1996). Oxidised lipids have also been found to be more deleterious to arterial health than native lipids themselves (Jadhav et al., 1996). There is little microbial activity in fats and oils due to the lack of water (List and Erickson, 1980). Thus, oxidation is most often the shelf life limiting factor for edible fats and oils.

Avocado oil is not recognised as significant on the world market (Eyres et al., 2001). Because of this reason, there have been very few studies performed on the oxidative stability of avocado oil. A literature search revealed that there have been only two papers published on the oxidation of avocado oil. The effect of antioxidants in refined, bleached and deodorised avocado oil and the oxidation of avocado oil under light and dark conditions have been studied (Werman and Neeman, 1986a; Werman and Neeman 1986b).

The aim of this research was:

- To examine the effect of prooxidant factors on the oxidation of EVAO
- To predict the oxidative shelf life of EVAO by examination of reaction kinetics under accelerated conditions
- To examine the effect of commercial antioxidants on the accelerated shelf life of EVAO

2 Literature Review

2.1 Avocado oil

Avocado fruit contain high levels of oil. In fact it is one of the few cultivated fruit in which oil is the main component on a dry weight basis (Brown, 1972). Oil is mainly contained in the fruit pulp. The cultivar 'Hass' has been shown to have one of the highest levels of pulp per whole fruit at around 75% w/w (Human, 1987). Hass avocados have the highest oil content in the fruit pulp compared to other cultivars (Human, 1987). Avocado oil has been successfully extracted from avocados by centrifugation, expulsion and solvent extraction (Buenrostro et al., 1986; Human, 1987; Werman and Neeman, 1987; Southwell et al., 1990; Bizimana, 1993).

Avocado oil has been successfully extracted from Hass avocado fruit on a commercial scale (Eyres et al., 2001). This oil was produced using centrifugal extraction equipment designed for extra virgin olive. Only warm water was used as an extraction aid; after centrifugation no other unit operations such as bleaching, steam stripping or chemical treatment were applied (Eyres et al., 2001). The resulting oil was called extra virgin, based on codex alimentarius guidelines that define the quality standards for extra virgin olive oils (Codex, 2000). These guidelines are:

- It has a free fatty acid level of less than 1%
- It contains no additives.
- It has a peroxide value of less than 20 milliequivalents of active oxygen/kg oil.
- It has superior organoleptic properties compared to refined oils with distinct flavours/odours
- It has less than 0.2 % moisture.
- It has less than 0.1 ppm Copper.

The fatty acid composition of extra virgin avocado oil (EVAO) produced in New Zealand has been determined by gas chromatography fatty acid methyl ester analysis (GC-FAME) and has also been compared to extra virgin olive oil (EVOO) produced in

New Zealand (Table 1).

Table 1. Fatty acid composition (percentage) of cold-pressed avocado oil and olive oil produced in New Zealand (Eyres et al., 2001)

Fatty Acid		NZ Avocado Oil	NZ Olive Oil
Palmitic	16:0	12.5 - 14.0	8.6 - 12.9
Palmitoleic	16:1	4.0 - 5.0	0.3 - 0.7
Stearic	18:0	0.2 - 0.4	2.1 - 2.8
Oleic	18:1	70 – 74	77.0 - 82.6
Linoleic	18:2	9.0 - 10.0	4.6 - 7.5
Alpha-linolenic	18:3	0.3 - 0.6	0.5 - 0.7
Arachidic	20:0	0.1	0.0 - 0.6
Gadoleic	20:1	0.1	0.0 - 1.4

As can be seen from Table 1, New Zealand EVAO has a similar fatty acid profile to EVOO, especially with respect to high levels of the monounsaturated fatty acid, oleic, and low levels of saturated fatty acids, palmitic and stearic. Also like EVOO, avocado oil contains many non-lipid components in very small quantities that have a large effect on appearance, taste, nutritional value and shelf stability (Rhamani and Csallany, 1998; Eyres et al., 2001). It is these minor components that make EVAO unique (Eyres et al., 2001).

Of the minor non-lipid components in avocado oil, it is perhaps chlorophyll that has the largest effect on both stability and appearance of the oil (Eyres et al., 2001). Chlorophyll is present at 15-60 ppm and imparts an emerald green colour to the oil (Human, 1987; Werman and Neeman, 1987). Although this colour may be beneficial to the consumer preference of avocado oil, chlorophyll is usually removed during processing of other vegetable oils since it has a detrimental effect on the oxidative stability (Human, 1987; Nawar, 1996).

2.2 Olive oil

EVOO it retains many of the properties intrinsic to the fruit from which it was extracted (Rhamani and Csallany, 1998). It is produced on a very large scale in many countries and is a well-known product worldwide. There have been many studies performed on the characteristics, specifically stability of EVOO and these studies may be relevant to EVAO (Minguez-Mosquera et al., 1990; Gutierrez-Rosales et al., 1992; Morales et al., 1994; Angerosa et al., 1999b; Reed et al., 2001).

The production of virgin oil from olives does not involve any refining or standardisation processes and the amounts of β-carotene, tocopherols, chlorophylls and polyphenols depend largely on cultivar and degree of maturity of the fruit and the method used for oil extraction (Rahmani and Csallany, 1998). These minor components may cause a lot of sample to sample variation in oil stability (Kiristakis et al., 1983). Model systems of oxidation and stability are less relevant to virgin oils since they do not take into consideration the possible interactions between non-lipid components and the oil. This is why oxidation studies on EVOO may be more applicable to EVAO (Kiristakis and Dugan, 1985; Rahmani and Csallany, 1998).

EVAO contains greater levels of chlorophyll than EVOO at 15-60 ppm vs. 4-8 ppm (Werman and Neeman, 1986; Human, 1987; Gutierrez-Rosales et al., 1992; Rahmani and Csallany, 1998). Chlorophyll destruction in olive oil is largely due to enzymes present during oil extraction (Minguez-Mosquera et al., 1990). High levels of chlorophyll make these oils more susceptible to photooxidation. Thus, the prevention of photooxidation in olive oil is of great importance to insure palatability and nutritional value (Rahmani and Csallany, 1998).

The effect of processing conditions on olive oil quality and stability were tested by Sifi et al. (2001). They concluded that the use of warm water during oil production can lead to the loss of water-soluble antioxidant compounds that may be of fundamental importance in ensuring that oils keep well during storage.

2.3 Quality loss and oxidation in oils

By far the major contribution of rancid flavour in oils comes from deterioration of the triglyceride molecules that make up the oil (Hamilton, 1994). Rancidity is considered to be the objectionable flavours and odours that result from the accumulation of lipid decomposition products (Gray, 1978). This can be due to either hydrolysis of the triglyceride to its fatty acid and glyceride components or due to oxidation of the oil, which forms a vast array of breakdown products (Hamilton, 1994).

Hydrolytic rancidity (or hydrolysis) in fats and oils may occur by enzyme action or by heat and moisture (Nawar, 1996). The enzyme most commonly responsible for this hydrolysis reaction is lipase (Galliard, 1994). Free fatty acid values in fresly extracted EVAO were found to be very low in contrast to fresh olive oil samples. Hence it was assumed that little hydrolysis occurred in avocado oil (Sherpa, 2000).

Fats and oils are oxidised when they come into contact with oxygen (Kiritsakis, 1998). Oxidation is of major concern to the food industry because it leads to the development of various off flavours and odours (Nawar, 1996). In addition, oxidative reactions can decrease the nutritional quality of food, especially through the loss in activity of the fat-soluble vitamins A and E and essential fatty acids (Hamilton, 1995; Kiritsakis, 1998). Oxidation in fats and oils is also partly responsible for the loss of pigments, specifically β-carotene and chlorophyll (Lawson, 1995).

It is generally accepted that the primary oxidation product in edible oils is a colourless, odourless intermediate known loosely as hydroperoxide (Hamilton, 1994). The hydroperoxide intermediate can be formed by two distinct types of oxidation – photooxidation (oxidation catalysed by light) and autoxidation (oxidation auto-catalysed by oxidation breakdown products) (Rawls and Van Santen, 1970). The reaction products of either pathways can auto-catalyse further autoxidation (Hamilton, 1994). Hydroperoxides can then break down to secondary oxidation products which are shorter chain carbon molecules such as aldehydes and ketones, that impart off-flavours and odours to the oil (Hamilton, 1994).

2.3.1 Autoxidation

Autoxidation is the most well known and commonly encountered route of oxidation during the storage of edible oils and the primary reactions are based on three phases; initiation, propagation and termination.

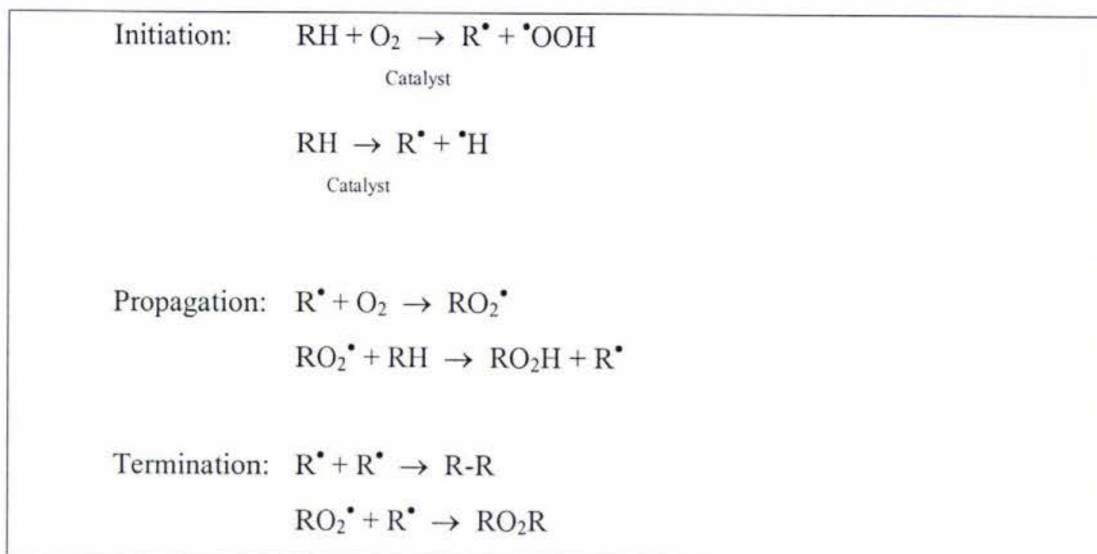


Figure 2. Reaction steps in autoxidation (Hamilton, 1994)

The initiation step for this reaction has a high activation energy, so autoxidation generally relies on free radicals and peroxides formed from photooxidation (Rawls and Van Santen, 1970). The initiation mechanism is not entirely understood but it produces highly reactive free radicals ($R^\bullet$) from lipid molecules (RH). This reaction is enhanced by external energy such as heat, or by chemical initiation involving metal cations. The initiation reaction is autocatalytic since free radical breakdown products will further catalyse initiation reactions (Hamilton, 1994).

Propagation-type reactions follow the initiation step, resulting in the autocatalytic production of hydroperoxides and their breakdown products (Hamilton, 1994). In contrast to the initiation step, the propagation phases of autocatalytic oxidation have very low activation energies (1-4 $kJmol^{-1}$) and will readily occur at low temperatures (Hamilton, 1994). Even refrigerator and freezer temperatures which normally slow or

inhibit microbial and enzymatic activity will not stop autoxidation at this stage. These reactions may also be catalysed by metal cations.

The final step is termination, where free radical propagation is terminated by the reaction of two free radicals, or by free radical and free radical acceptors and scavengers.

It is not the presence of hydroperoxides that gives rancid oil unpalatable flavours and odours, but the short chain secondary oxidation products – especially aldehydes, alcohols and ketones (Hamilton, 1994). Measuring the amount of hydroperoxides can give an indication of primary oxidation, while measuring and identifying the short chain breakdown products can give an indication of secondary oxidation and 'off-flavours' that may be encountered.

2.3.2 Photooxidation

Photooxidation is another oxidation mechanism that results in similar breakdown products to autoxidation, but follows a different pathway. This reaction mechanism is not autocatalysed by free radical and hydroperoxides intermediates like autoxidation, but by reactive intermediates formed as a result of exposure to a light source (Lee and Min, 1990).

Headspace oxygen is depleted when oils containing chlorophyll have been exposed to light (Lee and Min, 1988). An increase in peroxide value and conjugated dienes was also observed when chlorophyll-containing oils were exposed to light (Kiristakis and Dugan, 1985). This suggests that chlorophyll is a sensitiser in the photooxidation mechanism (Rawls and Van Santen, 1970; Usuki et al., 1984; Kiristakis and Dugan, 1985; Gutierrez-Rosales et al., 1992; Rahmani and Csallany, 1998). Since EVAO contains high levels of chlorophyll, photooxidation is rapid when exposed to daylight and fluorescent light (Werman and Neeman, 1986a).

During the initial stages of oxidation when hydroperoxides are scarce, photooxidation

rates are much higher than autoxidation (Rawls and Van Santen, 1970). Primary oxidation of bleached olive oil containing chlorophyll protected from light was only 15% of the comparable samples exposed to light as measured by the peroxide value (Kiristakis and Dugan, 1985). Even relatively low light levels can cause photooxidation (Endo et al., 1984a). Protection of edible oils from light can aid stability – soybean oil and hydrogenated soybean oil bottled in amber glass was found to have significantly better flavour scores than those bottled in clear plastic or glass after only four hours exposure to fluorescent light at 7535 lux (Warner and Frankel, 1987).

2.3.2.1 Photosensitisation type and identification

There are two types of photosensitisation (light sensitisation) which can cause oxidation in lipid compounds, type I and type II.

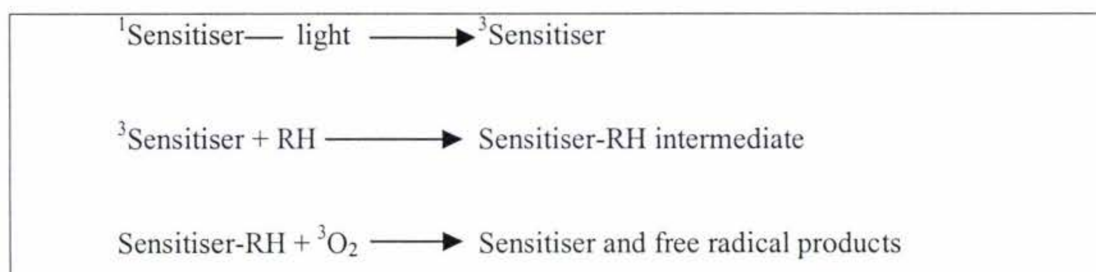


Figure 3. Type I photosensitisation (Chan 1977). Superscripts ^{1} and ^{3} refer to singlet or triplet state of that compound.

In type I sensitisation (Figure 3), the sensitiser becomes excited following light absorption and reacts directly with the lipid substrate to form intermediates. These intermediates react with ground state triplet oxygen to yield free radical and hydroperoxide oxidation products (Chan, 1977).

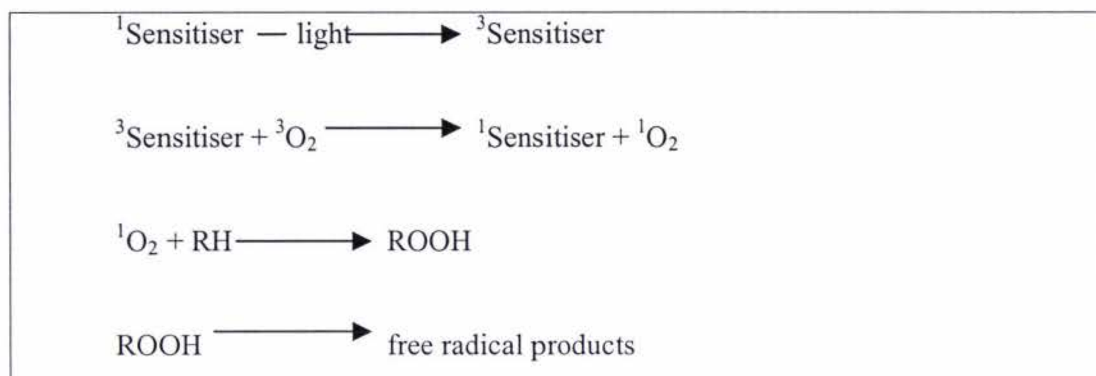


Figure 4. Type II photosensitisation (Chan, 1977). Superscripts ^{1} and ^{3} refer to singlet or triplet state of that compound.

The type II reaction is similar to type I photosensitisation except the excited sensitiser reacts with ground state triplet oxygen instead of the lipid (Chan, 1977; Korycka-Dahl and Richardson, 1978). More specifically, when a sensitiser absorbs energy in a type II reaction, it becomes an excited singlet sensitiser and changes to an excited triplet state. This triplet energy is transferred to ordinary triplet oxygen to produce singlet oxygen by triplet-triplet annihilation. Singlet oxygen can react directly with the double bonds of fatty acids by a symmetrical addition known as the "ene" reaction (Gutierrez-Rosales et al., 1992). This results in the formation of numerous hydroperoxide breakdown products (Jung and Min, 1991; Hall and Cuppett, 1993).

Analysis of the conjugation of the oxidation products and kinetics can be used to determine which type of oxidative reaction has taken place (Rawls and Van Santen, 1970; Chan, 1977). Chlorophyll has been found to sensitise type II singlet oxygen production in oils which characterises the oxidation of crude oils that have very high chlorophyll levels (Foote, 1968; Jung and Min, 1991; Hall and Cuppett, 1993). A basic understanding of chlorophyll and singlet oxygen chemistry is vital to understanding photooxidation.

2.3.2.2 Singlet oxygen chemistry

Many researchers have classified photooxidation as involving a singlet oxygen intermediate (Rawls and Van Santen, 1970; Clements et al., 1973; Frankel et al., 1982;

Whang and Peng, 1988). Singlet oxygen is the state of oxygen in which both free electrons are paired and the molecule has no magnetic moment (Korycka-Dahl and Richardson, 1978). Oxygen in the ground state with two unpaired electrons would exist in three closely grouped energy fields if placed in a magnetic field (triplet state) (Korycka-Dahl and Richardson, 1978).

Before singlet oxygen photooxidation was discovered, there was confusion over where initial oxidation products (hydroperoxides) arose from for the autoxidation reaction to take place (Rawls and Van Santen, 1970, Nawar, 1996). Triplet oxygen is very stable and is very unlikely to react directly with lipid double bonds or to cleave to form free radicals (Lee and Min, 1990). Singlet oxygen on the other hand reacts with unsaturated fatty acids to form hydroperoxides at least 1450 times faster than triplet oxygen (Rawls and Van Santen, 1970). This is because it can react directly with olefinic molecules while satisfying spin and energy conservation requirements. The hydroperoxides formed from singlet oxygen oxidation are able to decompose to free radical products that can initiate and catalyse autoxidation. Thus, a mechanism that could supply singlet oxygen such as photooxidation could explain the formation of the original hydroperoxides in lipids (Rawls and Van Santen, 1970)

A number of researchers have theorised that singlet oxygen is the intermediate of photosensitised oxidation and have contrasted it with autoxidation. This excited form of oxygen is very reactive; it has a very short lifetime and cannot be measured directly. However, the following evidence exists that supports singlet oxygen as the intermediate of chlorophyll sensitised photooxidation:

1. Oxidation products in methyl linoleate (a model lipid system) were similar for photooxidation and oxidation by artificially produced singlet oxygen, whereas they were different for autoxidised samples. The difference lay in the hydroperoxides – those produced by singlet oxygen and photooxidation contained a mixture of conjugated and non-conjugated products whereas autoxidised samples contained only conjugated hydroperoxides (Rawls and Van Santen, 1970). Clements et al. (1973) also found that in a model 1,4-diene, 4cis, 7cis-undecadiene system,

photooxidation gave the same initial products as singlet oxygen oxidation but autoxidation did not. A similar experiment performed in soybean oil also had the same results. Products formed in singlet oxygen oxidation and photooxidation were identical and these were different to products from autoxidation (Clements et al., 1973).

2. Rawls and Van Santen (1970) measured the order of effectiveness of photooxidation inhibitors by comparing oxidation products measured by thin layer chromatography (TLC) to give an approximate inhibition percentage. The order of inhibition effectiveness was the same as the order of reactivity of the inhibitor with singlet oxygen. This experiment was based on the singlet oxygen quenchers tetramethylethylene (TME), tetraphenylcyclopentadiene (cyclone), and diphenylisobenzofuran (DPBF). The order of singlet oxygen and photooxidation inhibition was TME < cyclone < DPBF.

3. Identical oxidation product distribution exists between chemically produced singlet oxygen oxidation and the chlorophyll-photosensitised oxidation of olefins and dienes. This was shown by identical distributions of alcohols and olefins as measured by gas chromatography (Foote, 1968). It was also shown that the reactive intermediates in both the photosensitised and singlet oxygen oxidation were identical in the stereoselectivity of substrate attack and electrophilicity.

Most photosensitisers including protoporphyrin and methylene blue can be completely inhibited by known singlet oxygen quenchers, suggesting their mechanism is due entirely to singlet oxygen. However, chlorophyll could not be fully inhibited in the same way. Chlorophyll could only be 80% inhibited by singlet oxygen quenchers (Rawls and Van Santen, 1970).

The peroxides formed through oxidation by singlet oxygen photooxidation undergo scission to free radical species much like those formed from autoxidation. However, unlike hydroperoxides formed from free radical attack, those formed from singlet oxygen can decompose at room temperature (Kiristakis et al., 1983). This can cause

acceleration in the rate and ultimate level of peroxide formation via subsequent autoxidation (Kiristakis and Dugan, 1985; Rahmani and Csallany, 1998).

2.3.2.3 Biological consequences

Lipid breakdown products such as free radicals can cause further breakdown of lipids, but can also bring about cellular damage to biological material (Frankel, 1984). In humans, chemical carcinogenesis may result from the enzymatic or non-enzymatic oxidation of chemical agents into reactive intermediates formed either from stable free radicals or via singlet oxygen and hydroxide radicals by complex metal catalysis (Frankel, 1984). Also, photosensitising dyes, analogous to chlorophyll, can sensitise the oxidative destruction of nucleic acids and it is this mechanism which may lead to the development of skin cancer in humans (Foote, 1968). However, protective enzymes such as peroxidase, catalase and superoxide dismutase can remove different species of activated oxygen that promote lipid oxidation. The relatively low intracellular concentration of oxygen is another defence mechanism against lipid oxidation (Frankel, 1984).

Plant materials also contain substances that have natural antioxidative capabilities. Carotenes are well-known antiphotooxidants (Warner and Frankel, 1987; Jung and Min, 1991) and tocopherols are good antiautoxidants (Niki, 1996).

2.3.3 Chlorophyll breakdown and stability in oils

In a study performed on avocado oil oxidation it was shown that the chlorophyll content in EVAO is rapidly reduced on exposure to daylight and fluorescent light. After 27 days of exposure to a 40 W fluorescent lamp, the chlorophyll content of the oil dropped to approximately 18% of its original value (Werman and Neeman, 1986a)

Chlorophyll can be rapidly broken down by hydroperoxides, hydroperoxide breakdown products, free fatty acids, UV light (<300nm wavelength), visible light at ~650nm, singlet oxygen and heat (Rawls and Van Santen, 1970; Endo et al., 1984; Usuki et al.,

1984; Endo et al., 1985a; Werman and Neeman, 1986a; Baardseth and Von Elbe, 1989). Ethylene, a plant senescence hormone, was found not to accelerate chlorophyll breakdown (Baardseth and Von Elbe, 1989). Other than exposure to free fatty acids, all of the other factors that lead to chlorophyll degradation are due directly to light or its influence on chlorophyll. In fact, degradation is so rapid in lit conditions that degraded chlorophyll no longer participates in the sensitiser role in the latter stages of photooxidation (Kiristakis and Dugan, 1985). Thus it is the stability of chlorophyll and its decomposition products that are assumed to be responsible for the different prooxidant activities of the chlorophyll pigments which include chlorophyll *a* and *b* and pheophytin *a* and *b* (Endo et al., 1984a; Endo et al., 1984b). The structure of chlorophyll *a* and *b* and pheophytin *a* and *b* are shown in Figure 5.

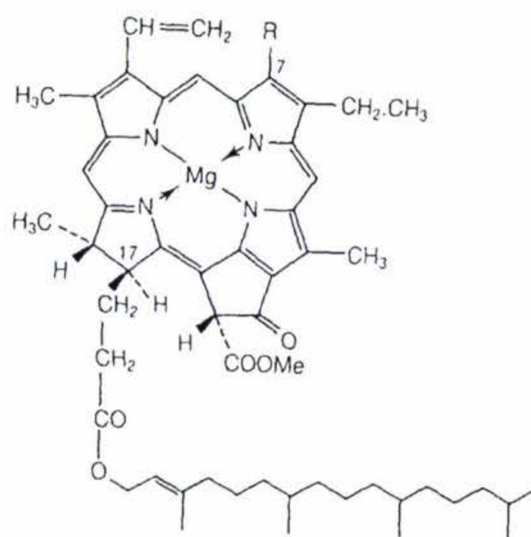


Figure 5. Chemical structure of chlorophyll *a* where R = CH_3 and chlorophyll *b* where R=CHO. Structure also corresponds to pheophytin *a* and *b* when magnesium is absent (Hendry, 1993).

The presence of pheophytin and pheophorbide, magnesium free derivatives of chlorophyll, may be as important or even more important than that of chlorophyll (Endo et al., 1984a; Endo et al., 1984b; Usuki et al., 1984; Kiristakis and Dugan, 1985). These chlorophyll derivatives are not formed from chlorophyll in the oil, but are extracted from the fruit during oil extraction (Minguez-Mosquera et al., 1990). The reasoning

behind the higher prooxidant activity of these two compounds is that they have similar singlet oxygen producing characteristics to chlorophyll but are in fact more stable to light related decomposition. This is shown by their insensitivity to oxygen during light irradiation (Endo et al., 1984a; Usuki et al., 1984). It is for this reason that the pheophytin/pheophorbide content should be noted alongside chlorophyll when considering the oxidative stability of edible oils. However, the absence of pheophorbide in edible oils has been confirmed (Usuki et al., 1984).

The pheophytin content is especially important for crude edible plant oils such as virgin avocado and olive oil since they have been found to have greater amounts of pheophytins than chlorophylls according to fluorospectrometric analysis (Endo et al., 1984a). Refined edible plant oils contain much higher proportions of chlorophyll than pheophytins (Usuki et al., 1984). This suggests that the RBD process removes most of the pheophytin pigments. Pheophytin was also found to be more stable to higher temperatures than chlorophyll; at 30°C the absorption maxima of chlorophyll disappeared and at 50°C pheophytin was broken down as well (Endo et al., 1985a).

Chlorophyll only breaks down to pheophytin or pheophorbides under highly acidic conditions or during the ripening of olives (Minguez-Mosquera et al., 1990). It is however expected to break down to colourless, low molecular weight compounds (Endo et al., 1984a).

2.3.4 Effect of enzymes on oil quality

2.3.4.1 Lipoxygenase

Lipoxygenase activity is associated with the destruction of carotenoids and oxidative instability (Eskin and Grossman, 1977; Georgalaki et al., 1998). Thus, the inactivation of lipoxygenase can prevent colour changes associated with the conversion of chlorophylls to pheophytins, chlorophyll destruction and off-flavour development in lipids (Minguez-Mosquera et al., 1990). In fact, the bleaching of chlorophyll in aqueous extracts of soybean oil was one of the first identified characteristics of lipoxygenase

(Eskin and Grossman, 1977).

Olives contain a significant quantity of lipoxygenase (Georgalaki et al., 1998). All EVOO samples tested in one study showed detectable amounts of protein, as well as lipoxygenase and polyphenoloxidase activity (Georgalaki et al., 1998). The activity of these water-soluble enzymes however could not be correlated to the moisture content of the samples (Angerosa et al., 1999b).

Unlike the process of autoxidation, oxidation catalysed by lipoxygenase is highly specific and produces 6-carbon aldehydes that give olive oil its desirable and characteristic aroma (Eskin and Grossman, 1977; Anonymous, 1999b). Lipoxygenase is released during tissue disruption (milling) and is active during malaxation of the fruit pulp during olive oil production (Angerosa et al., 1999b). Milling fruit with no stones produced a greater content of 6-carbon aldehydes since more lipoxygenase was released during milling as a result of more serious cellular disruption (Angerosa et al., 1999a). Malaxing the pulp for longer periods also increased the content of 6 carbon aldehydes since the lipoxygenase was able to act for longer.

The mechanism of lipoxygenase does not involve a free radical chain process like autoxidation. The intermediate formed in the lipoxygenase reaction is a close analogue to that of singlet oxygen, but direct evidence that implicates any enzyme in actual singlet oxygen production is sparse (Eskin and Grossman, 1977; Korycka-Dahl and Richardson, 1978).

Because of the nature of lipoxygenase, antiautoxidants tend to inhibit its activity, especially α-tocopherol, synthetic hindered phenols, polyphenolic antioxidants as found in olive oil and epicatechin (Eskin and Grossman, 1977; Marcus et al., 1988). The activity of epicatechin is especially important for avocados since the decrease in epicatechin content during the ripening of avocado fruit may result in the enhanced lipoxygenase activity in these fruits (Marcus et al., 1988). Purified avocado lipoxygenase from this study showed an optimum pH of about 6 (Marcus et al., 1988). Adjustment of the pH to a more acidic level has proved to be an effective method for

inhibiting lipoxygenase, especially when used in combination with heat treatments (Eskin and Grossman, 1977).

2.3.4.2 Lipase

The enzyme lipase is responsible for catalysing hydrolysis of ester bonds in lipids, resulting in the liberation of free fatty acids from the triglyceride molecule (Nawar, 1996). Glycerides, monoglycerides or diglycerides are also formed in this reaction as the remnants of the triglyceride molecule (Hamilton, 1994). Free fatty acids formed in this reaction have a greater susceptibility to oxidation than their triglyceride precursors (Hamilton 1994; Nawar 1996; Reed et al., 2001). Free fatty acids can be easily measured and the percentage of free fatty acids in an oil gives a good indication of lipid hydrolysis (AOCS, 1993).

2.3.4.3 Fruit Quality

Fruit quality also has a strong bearing on the final quality of the oil extracted from it (Kiritsakis, 1998). When fruit of poor quality is processed in the manufacturing of olive oil, the oil extracted is also of poor quality, having undergone significant hydrolysis and subsequent oxidation due to the action of lipase in the fruit (Kiritsakis, 1998). This oil must be refined, bleached and deodorised for use in the food industry and is of much lower value (Kiritsakis, 1998).

A similar phenomenon occurs when processing avocado fruit of poor quality for oil. The activity of lipase in avocado has been presumed to be low compared to that in olives (Sherpa, 2000). However when poor quality fruit are used in the extraction process, the free fatty acid content (a measure of triglyceride breakdown) will be high and the oil will have a strong rancid smell (Human, 1987). Therefore, fruit quality must also be examined with respect to oil quality (Woolf, 2001).

2.3.4.4 Exogenous processing enzymes

Previously, studies on enzyme processing aids for olive (and avocado) oil production were mainly aimed at enhancing extraction yields, but now research is primarily conducted to improve product quality.

Ranalli and De Mattia (1997) recently studied a commercial enzyme mix that consisted mostly of pectinase and cellulolytic hemicellulolytic enzymes. These enzymes were mostly common in olive fruit but destroyed during the extraction process. Thus, addition of this enzyme mix replaced and enhanced enzymes that were present naturally in the fruit prior to extraction. The enzyme mixture was chosen because of its ability to degrade the walls of oil bearing cells. It also had a similar affect on the colloidal systems in the paste. In practical terms, this would help release the small fraction of oil remaining in the cytoplasm and have a positive effect on the rheological characteristics of the paste for processing. Consequently, more oil was recovered from the vegetable water due to disruption of the emulsion (Ranalli and De Mattia, 1997). In this study, the enzyme was used at 0.03 % vol./wt after being diluted 1:9 in lukewarm water. Olive oil produced using the enzyme exhibited higher total polyphenol contents, which has been associated with a higher resistance to autoxidation (Gutfinger, 1981). Use of the processing enzyme did not affect the fatty acid composition of the oil significantly but did lead to about a 5% higher overall output. Because of this, the resulting husk was characterised by a lower residual oil and water content due to a more effective separation of phases from the olive paste.

Freitas et al. (1998) used 0.05 % w/w of pectinolytic enzymes in avocado paste to aid in the extraction of avocado oil. It was concluded from their research that the benefits of utilising enzymes in the extraction of oil from plant matter lies in the mild operating conditions, which can result in a higher quality product and the added advantage of energy savings.

2.3.5 Oxygen removal

Since the formation of singlet oxygen during exposure to light requires the presence of triplet oxygen as a substrate, the presence or lack of oxygen also strongly influences the rate of singlet oxygen formation and subsequent photooxidation (Korycka-Dahl and Richardson, 1978). Dissolved oxygen in edible oil should therefore be considered as well as headspace oxygen, especially since oxygen is much more soluble in non-polar substances than water.

Increasing headspace oxygen content has little effect on accelerating autoxidation at ambient temperature, but at higher temperatures, the effect of oxygen presence can become considerable (Ragnarsson and Labuza, 1977).

Oxygen contact can be minimised in oil products at several stages. These include managing oxygen pickup during bottling, removal of oxygen using special oxygen scavengers bound to packaging material and sparging following oil production using bubbles of nitrogen gas (Rooney et al., 1981; Berger, 1994; Tsiadi et al., 1999). In fact the long term storage of edible oils requires almost complete removal of headspace and dissolved oxygen down to well below 2% v/v (Rooney et al., 1981).

The key to managing oxygen pickup during bottling is to ensure that dissolved oxygen and carbon dioxide levels are known prior to bottling (Rooney et al., 1981). From this basis, the use of oxygen removing techniques may be considered. One such technique is the use of oxygen scavengers in packaging materials. These have the ability to reduce oxygen to the detection limit of 0.03 %, making them particularly sensitive in oxygen sensitive foods such as oil (Rooney et al., 1981). Oxygen scavenging packaging materials work similarly to photosensitisers – ground state oxygen is converted to singlet oxygen by a film-immobilised dye. The singlet oxygen then reacts with an acceptor, also immobilised in the film and is thereby consumed (Rooney et al., 1981). This type of singlet oxygen acceptor may potentially be able to remove singlet oxygen formed by photooxidation.

Kiristakis and Dugan (1984) found that a rancid odour was not formed in olive oil where there was limited oxygen in the headspace. Reed et al. (2001) also reported that secondary oxidation was limited by lack of oxygen in sealed bottles. Reduction of oxygen level in the headspace may be achieved by nitrogen flushing of the bottle.

2.3.6 Nitrogen Sparging

An effective way of removing dissolved oxygen from oil is by sparging it with nitrogen. This involves forcing nitrogen through a sintered plate into oil following production.

Producing small, spherical bubbles during sparging creates a larger surface area per unit volume and therefore provides an increased mass transfer compared with larger bubbles. Bubbles formed from being forced through a sintered plate are spherical at the formation stage but change to ellipsoidal upon attachment due to the viscous resistance of the oil (Tsiadi et al., 1999). Increased gas velocity through smaller pores resulted in interference between bubbles at the stage of formation such that gas from a new forming bubble entered an older one, causing its expansion. Once formed these bubbles did not coalesce in sunflower oil and the same can be expected for other vegetable oils (Tsiadi et al., 1999). Since bubble size is related to the viscosity of the oil, bubble size increased with temperature (Tsiadi et al., 1999).

Sparging oil with nitrogen at low temperatures can also strip oil of surface active contaminants (Tsiadi et al., 1999). This is because they will move to the oil-nitrogen interface where they are more stable, then they are carried to the surface of the oil by the buoyancy of the bubble. When the bubbles reach the surface, adsorbed contaminants are flocculated and are removed. Removing surface-active contaminants is beneficial since they are able to solubilise water-soluble components such as enzymes, which may have an adverse affect on oil quality. Sunflower seed oil was also found to have significant quantities of wax that dissolved in the oil when heated to 50-70°C (Tsiadi et al., 1999). Since the temperature of the nitrogen used to sparge the oil was significantly lower than this, sparging caused rapid nucleation and subsequent precipitation of the wax (Tsiadi et al., 1999).

2.4 Antioxidants

Natural oils and fats from vegetable sources contain minute amounts of substances capable of inhibiting oxidation to some extent called antioxidants (Lawson, 1995). Photooxidation is not controlled by antioxidants commonly used to control oxidation (Kiristakis and Dugan, 1985). Hence, at this point two types of antioxidants shall be defined. Antiphotooxidants are those antioxidants that inhibit photooxidation and antiautoxidants are the better known antioxidants that inhibit autoxidation.

2.4.1 Antiautoxidants

Traditional food antioxidants are those substances which quench or inhibit the free radical autoxidation of lipids. These substances may be naturally occurring such as Vitamin E in vegetable oils, or artificial such as propyl gallate (PG), butylated hydroxyanisole (BHA) or butylated hydroxy-toluene (BHT) (Coppen, 1994). Since the two types of lipid oxidation outlined in this review involve distinctly different mechanisms, the antioxidants operate differently also; an antiautoxidant will not specifically work well as an antiphotooxidant and vice versa.

Antiautoxidants are of two types – primary or secondary. Primary antioxidants include polyhydric phenolic and hindered phenolic compounds, which function mainly as electron or hydrogen donors (Rajalakshmi and Narisimhan, 1996). Their antioxidative function is by converting free radicals into more stable products, thus interrupting the free radical chain reaction. This occurs during free radical propagation (Figure 2) and is stabilised by low energy resonant structures of the antioxidant (Hamilton, 1994). The hindered phenols are stable at high temperatures encountered in food processing as well as having carry-through properties that remain in baked or fried products (Niki, 1996). Commonly encountered primary antioxidants used in food products are propyl gallate, butylated hydroxyanisole, butylated hydroxytoluene, tert-butyl hydroquinone (TBHQ) and tocopherols.

Secondary antioxidants are a more diverse group of compounds that act synergistically

with primary antioxidants. They may function as electron or hydrogen group donors to primary antioxidant radicals thereby regenerating the primary antioxidant (Madhavi et al., 1996). Other mechanisms are metal chelation (as metal ions can act as prooxidant); providing an acidic environment for primary antioxidants to function better; oxygen scavenging or singlet oxygen quenching. Singlet oxygen quenchers such as β-carotene will be discussed separately under antiphotooxidants.

2.4.2 Primary antiautoxidants

2.4.2.1 Tocopherols (vitamin E)

The tocopherols are a chemically related group of tocols and tocotrienols that are widely distributed in plant tissues (Eyres, 2000). They are known more commonly in food and nutrition as vitamin E. Since the human body cannot manufacture vitamin E it must be taken in with food ingested or as a supplement (Eyres, 2000). The tocopherols exist as eight isomers: α-, β-, γ-, δ- tocols and trienols, each with varying antioxidant activities.

Table 2. Contents of Tocopherols/Trienols in some common vegetable oils (Madhavi et al., 1996)

Vegetable oils	Tocopherols (mg)				Tocotrienols (mg)			
	alpha	beta	sigma	gamma	alpha	beta	sigma	gamma
Coconut	5-10	-	5	5	5	Trace	1-20	-
Cottonseed	40-560	-	270-410	0	-	-	-	-
Maize grain	60-260	0	400-900	1-50	-	0	0-240	0
Olive	1-240	0	0	0	-	-	-	-
Palm	180-260	trace	320	70	120-150	20-40	260-300	70
Peanut	80-330	-	130-590	10-20	-	-	-	-
Rapeseed	180-280	-	380-590	10-20	-	-	-	-
Safflower	340-450	-	70-190	230-240	-	-	-	-
Soybean	30-120	0-20	250-930	50-450	0	0	0	-
Sunflower	350-700	20-40	10-50	1-10	-	-	-	-
Walnut	560	-	590	450	-	-	-	-
Wheatgerm	260-1200	660-810	260	270	20-90	80-190	-	-

Tocopherols are considered natural primary antioxidants. The antioxidant activity of tocopherols is mainly due to their ability to donate their phenolic hydrogen to lipid free radicals (Kamal-Eldin and Appelqvist, 1996). They act by inhibiting the initiation and propagation of free radicals, the classic mechanism of lipid oxidation (Niki, 1996; Eyres, 2000). They have limited carry through properties in cooking and are less effective than BHT, BHA, TBHQ or PG (Chen et al., 1992). However, they do have excellent carry through properties in oil refining (Clements et al., 1973) and so have a strong influence on the initial oxidative stability of vegetable oils, especially virgin olive oil (Kiristakis et al., 1983; Frankel, 1993; Rahmani and Csallany, 1998). Various tocopherols can also be extracted and isolated naturally from the sludge obtained from deodorisation of vegetable oils and fats (Madhavi et al., 1996). α-Tocopherol and its acetate form can also be created synthetically. These products are designated as *dl*-α-tocopherol and *dl*-α-tocopherol acetate (Madhavi et al., 1996).

Natural tocopherols are insoluble in water but are soluble in fats and oils. α-Tocopherol is the most abundant of all tocopherols and its biological activity is twice that of the β and γ homologues and 100 times that of the δ homologue (Madhavi et al., 1996). Tocopherols can function synergistically with other primary and secondary antioxidants. A combination of tocopherol and other antioxidant synergists are often used together since tocopherols usually contribute to an undesirable or foreign flavour of their own (Madhavi et al., 1996).

Under physiological conditions i.e. within living cells, the antioxidant activity of tocopherols is in the order of $\alpha > \beta > \gamma > \delta$, the same as their biological activity (Madhavi et al., 1996). However, the order of antioxidant efficacy is reversed when these tocopherols are used in pure oils as antioxidants; $\delta > \gamma > \beta > \alpha$ (Jung et al., 1991). This can be explained by the relative oxidative stabilities of the tocopherols themselves, which are in the same order. The lower the individual oxidative stability of each tocopherol, the lower the optimum concentration of that tocopherol for maximum oxidative stability and the lower the overall effectiveness (Jung et al., 1991). The less stable tocopherols also have a stronger prooxidant effect when oxidised (Jung and Min, 1992). This data is shown in Table 3

Table 3. Relative abilities of the 4 tocopherol groups in different conditions

Efficacy	Physiological antioxidative abitilty	Antiautoxidant Ability	Individual oxidative stability	Prooxidant effect when oxidised	Antiphotooxidant ability
Strongest	alpha	delta	delta	alpha	alpha
	beta	gamma	gamma	beta	beta
	gamma	beta	beta	gamma	gamma
Weakest	delta	alpha	alpha	delta	delta

Although α-tocopherol has the lowest activity of all tocopherols as an antioxidant in oils, it is the most commonly used because it is the most abundant and can easily be synthesised as *dl*-α-tocopherol acetate, a mixture of four racemates (Madhavi et al., 1996).

The optimum concentration of α-tocopherol in stripped soybean oil undergoing autoxidation was found to be 100ppm; above this level it had a prooxidant effect (Jung et al., 1991; Kamal-Eldin and Appelqvist, 1996). This was because oxidised tocopherols catalysed peroxide formation that accelerated autoxidation (Jung and Min, 1992). Therefore, removal of both excess tocopherols and oxidised tocopherol compounds, may be beneficial to autoxidative stability (Jung and Min, 1992).

The limitation of tocopherol as an antioxidant lies with its own susceptibility to oxidation. Therefore, it is recommended that it be used with a synergistic antioxidant such as β-carotene, which has been found to protect α-tocopherol well against singlet oxygen attack (Warner and Frankel, 1987). Tocopherol levels in extra virgin olive oil produced by both pressing and centrifugation techniques remained fairly stable after 6 months in cool and dark conditions which suggested they were protected by other antioxidants – probably polyphenols that are unique to olive oil (Sifi et al., 2001).

Unlike other antiautoxidants, tocopherols have a small positive affect on stability towards photooxidation (Kiristakis and Dugan, 1985; Jung et al., 1991). Of the four tocopherol groups, α-tocopherol was found to be the best antiphotooxidant in the photosensitised oxidation of soybean oil, β-tocopherol second, γ-tocopherol next,

then δ-tocopherol. Tocopherols in bleached olive oil with added chlorophyll were rapidly destroyed (Kiristakis and Dugan, 1985). This was because tocopherols tend to quench singlet oxygen quite effectively, but undergo relatively rapid oxidation themselves, causing them to lose singlet oxygen quenching properties and act as prooxidants (Carlsson et al., 1976; Jung and Min, 1992; Kamal-Eldin and Appelqvist, 1996). Thus if reactions with singlet oxygen were to run for long periods, γ- and δ-tocopherols may prove to be more effective than α-tocopherol due to their higher stability to singlet oxygen (Kamal-Eldin and Appelqvist, 1996). Although the performance of α-tocopherol in quenching singlet oxygen is approximately 50 times less than β-carotene and 200 times less than lutein, it is still able to deactivate 40-120 molecules of singlet oxygen before it is deactivated (Jung et al., 1991; Kamal-Eldin and Appelqvist, 1996).

2.4.2.2 Propyl Gallate

Propyl gallate (PG) belongs to the phenol family and is a component of many antioxidant mixtures. It has much better solubility in water than in oil thus is very effective at the oil-water interface in oils (Madhavi et al.,1996). PG does not have significant carry through properties but is very effective in anhydrous fats and oils. Addition of PG to crude vegetable oils before refining results in higher oxidative stability than without (Madhavi et al., 1996). In research performed on the stability of refined avocado oil, it was found that PG improved both the oxidative and colour stability of the oil when stored in the dark at 60°C (Werman and Neeman, 1986a). However, in photooxidative conditions i.e. under light, PG had no effect on reducing photooxidation of the oil (Werman and Neeman, 1986b).

2.4.2.3 Butylated hydroxyanisole

Butylated hydroxyanisole (BHA) is a phenolic compound and is commonly used as an artificial food antioxidant. BHA is very soluble in fats and oils and exhibits excellent residual activity in baked and fried foods, and this is its main use (Madhavi et al., 1996). BHA functions poorly alone but performs well with other primary antioxidants as

synergists, and is often used as a reference when testing novel antioxidants (Madhavi et al., 1996). Like PG, when BHA is added to crude oil before refining a higher oxidative stability is achieved.

BHA does not inhibit photooxidation of oils due to its lack of ability to scavenge singlet oxygen (Kim et al., 2000). Hydroperoxide formation due to photooxidation in virgin olive oil was almost unaffected by BHA addition (Kiristakis et al., 1983). High concentrations of BHA offered little protection against singlet oxygen in both purified soybean oil and in virgin olive oil (Kiristakis et al., 1983; Yasei et al., 1996).

2.4.2.4 Butylated hydroxytoluene

Butylated hydroxytoluene (BHT) is also an artificial primary phenolic antiautoxidant and has very similar properties to BHA. However, it is slightly less effective than BHA except in lard, and has less carry-through properties (Madhavi et al., 1996; Coppen, 1994). Also like BHA, BHT acts synergistically with other primary antioxidants. BHT can impart a phenolic odour to fats at concentrations higher than 0.02% (Madhavi et al., 1996). BHT is also less effective in photooxidised systems. It performs poorly in photooxidative oil systems since it cannot effectively scavenge singlet oxygen. This means that it has little effect on hydroperoxide formation due to photooxidation (Kiristakis et al., 1983; Yasei et al., 1996; Kim et al., 2000).

2.4.2.5 Tert-butyl hydroquinone

Tert-butyl hydroquinone (TBHQ) is an artificial phenolic primary antiautoxidant that is very effective in stabilising both crude and refined polyunsaturated vegetable oils (Madhavi et al., 1996; Coppen, 1994). It has good solubility in a wide range of fats and oils and has greater or equal activity to PG, BHA and BHT (Madhavi et al., 1996). Unlike BHA and BHT though, it does not impart any colour or odour to fats or oils and can act synergistically in vegetable oils with tocopherols. It has no carry through in baked products but does carry through in fried foods. TBHQ is often effective in situations when PG, BHA and BHT are not, such as in crude vegetable oil (Madhavi et

al., 1996). Like other antiautoxidants, TBHQ is not effective as an antioxidant when oils are photooxidised due to its lack of ability to quench singlet oxygen (Kim et al., 2000; Yasei et al., 1996).

2.4.2.6 Polyphenols

Olive oil contains high levels of polyphenols that have antiautoxidant activity, making the oil very resistant to oxidation (Gutfinger, 1981). In fact a linear relationship was found between polyphenol content and the oxidative stability of EVOO during storage at 60°C (Gutfinger, 1981). The efficacy of these natural antioxidants was shown when virgin olive oil stored in the dark at room temperature had an oxidation rate so low that peroxides were actually being destroyed faster than they were being formed (Gutierrez-Rosales et al., 1992). Even after 22 weeks, the peroxide value of EVOO was essentially unchanged (Kiristakis et al., 1983). Polyphenols also work poorly as antiphotooxidants.

2.4.3 Secondary antiautoxidants

2.4.3.1 Ascorbic acid and ascorbyl palmitate

Ascorbic acid acts as an oxygen scavenger in food products and in bacterial and mammalian systems (Rooney, 1983). Due to its relative insolubility in fats and oils, it is commonly used in the form ascorbyl palmitate for these food products. However even ascorbyl palmitate is still relatively insoluble in fats and oils at its effective usage rate of around 500 ppm (Madhavi et al., 1996). It is highly effective in synergistic mixtures with primary antioxidants for protecting both fat-soluble nutrients like vitamin A and vitamin E (Buettner and Jurkiewicz, 1996). Ascorbyl palmitate is generally regarded as safe (GRAS) and has no limitations in food use (Lee et al., 1997). This is because metabolism of ascorbyl palmitate forms ascorbate and palmitic acid (Lee et al., 1997).

Ascorbyl palmitate was found to be a more effective as an antiautoxidant than both BHT and BHA in vegetable oils and gave even greater extended protection when used with PG or naturally occurring tocopherols (Lee et al., 1997). An addition to its ability

to scavenge free radical oxygen and hydroxide species, it is also able to scavenge singlet oxygen by a quenching mechanism (Bodannes and Chan, 1979; Lee et al., 1997). This singlet oxygen quenching ability was found to be about 10 times greater than α-tocopherol, thus significantly reducing photosensitised oxidation in linoleic acid and soybean oil (Lee et al., 1997). However this activity was still about 10 times less than that of β-carotene although, unlike β-carotene, ascorbyl palmitate imparts very little colour or flavour to oil and has obvious antiautoxidative properties as well (Lee et al., 1997).

2.4.3.2 Citric acid

Citric acid is a natural constituent of plants and animals and one of the most versatile and highly used organic acids in foods and pharmaceuticals (Madhavi et al., 1996). It is highly soluble in water but not in fats and oils. It can be readily solubilised in fats and oils from propylene glycol solutions (Madhavi et al., 1996). Citric acid is widely used as a food acid. It also has metal chelating properties and is an excellent synergist with antiautoxidants. This means it a common constituent of many commercially available antiautoxidants (Chang et al., 1977).

Citric acid is widely used in oil refineries, where it is added during the cooling cycle of deodorisation to act as a synergist with other antioxidants, natural or added (Lindsay, 1996). It also has a synergistic effect as an antiphotooxidant. When used in soybean oil exposed to light, only 5-10ppm β-carotene was required for flavour stability whereas 20 ppm β-carotene was required for the stability without citric acid (Warner and Frankel, 1987). This is especially important for β-carotene because it imparts undesirable colour odour at levels of 20 ppm and above (Lee et al., 1997). However, more volatile oxidation products were produced where citric acid was present with β-carotene compared to β-carotene by itself (Warner and Frankel, 1987). Citric acid alone was found not to protect virgin olive oil against photooxidation at room temperature (Kiristakis et al., 1983).

2.4.3.3 Chlorophyll

In studies performed with a model system containing methyl linoleate, rapeseed oil and soybean oil, it has been shown that when stored in the dark, chlorophyll can act as an antiautoxidant (Endo et al., 1985a). This observation of chlorophyll acting as an antioxidant has also been witnessed in avocado oil (Werman and Neeman 1987). The antioxidant activity of chlorophyll is in strictly dark conditions only, since it acts as a strong sensitiser for photooxidation in the light (Rawls and Van Santen, 1970).

In a study of chlorophyll as an antiautoxidant in methyl linoleate it was found that at a concentration of 2.2×10^{-8} mol/g, chlorophyll *a* had very good antioxidant activity followed by BHT, chlorophyll b, then the pheophytins (Endo et al., 1985a). In fact, both chlorophylls and pheophytins inhibited the production of conjugated dienes in the dark at 30°C. When stored in dark conditions, EVOO samples containing added chlorophyll had slightly better oxidative stability than those containing only natural chlorophyll (Gutierrez-Rosales et al., 1992). Results were similar for rapeseed oil and soybean oil, however naturally occurring tocopherol tended to antagonise the effect of chlorophyll acting as a lipophyllic antiautoxidant. Decomposition of chlorophyll in this model system occurred much faster at 50°C than 30°C (Endo et al., 1985a). A possible reason for this is that chlorophyll is not only bleached by hydroperoxides formed during the oxidation of oils, but also by heat. Endo et al (1985a) claim that chlorophyll in vegetable oils can act as an antioxidant, preventing oxidative deterioration if the oil is not exposed to light and is stored at low temperatures.

In a follow-up paper, Endo et al., (1985b) suggest a likely mechanism for chlorophyll acting as an antiautoxidant, as shown in Figure 6. They suggest chlorophyll acts as a hydrogen donor, either to reduce free radicals and break the free radical propagation cycle, or to recharge primary antioxidants. Since hydroperoxides tend to break down chlorophyll fairly rapidly, chlorophyll shows antioxidant activity only in the initial stage of autoxidation before hydroperoxides are formed.

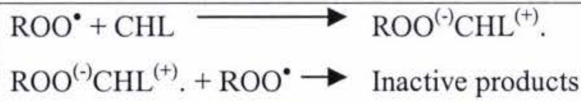


Figure 6. Antioxidant mechanism of chlorophyll (Endo et al., 1985b)

2.4.4 Antiphotooxidants

Antiphotooxidants are usually the lesser known antioxidants. These substances may also be naturally occurring or artificial. Antiphotooxidants act by quenching singlet oxygen, the intermediate in type II photooxidative reactions (Warner and Frankel, 1987; Jung and Min, 1991; Hall et al., 1994; Clifford et al., 1998). They return the excited singlet oxygen to its much less reactive triplet ground state so that it cannot react with fatty acid double bonds. It had been hypothesised that the mechanism of some antiphotooxidants may actually be to quench excited triplet chlorophyll before it can react with oxygen, but several recent kinetic studies have disproved this (Lee and Min, 1988, Lee and Min, 1990; Jung and Min, 1991, Rahmani and Csallany, 1998)

2.4.4.1 Carotenoids

Carotenoids are naturally occurring antiphotooxidants that occur widely in all plants and are commonly used as a colourant in the food industry (Deshpande et al., 1995). Beta-carotene is very sensitive to atmospheric oxygen, which can oxidise the conjugated double bond structure of the molecule. However, the stability of carotenoids improves on dissolution or suspension in lipids (Madhavi et al., 1995).

The antiphotooxidative activity of carotenoids comes from the long carbon backbone structure containing conjugated carbon-carbon bonds. It is in fact these conjugated carbon bonds that offer antiphotooxidant activity since their stable resonance structures are able to transform singlet oxygen back into its non-excited triplet oxygen state (Jung and Min, 1991).

Evidence supporting the singlet oxygen quenching ability of β-carotene, perhaps the

most common carotenoid, comes mostly from lipid photooxidation studies. In several studies where soybean oil with added chlorophyll was exposed to light irradiation, increasing the β-carotene content from 0-20ppm decreased headspace oxygen depletion, hydroperoxide formation, and conjugated diene formation (Fakourelis et al., 1987; Lee and Min, 1988; Lee and Min, 1990; Jung and Min, 1991). When stored in dark conditions, β-carotene did not have any effect on the oxidation of soybean oil (Lee and Min, 1988; Jung and Min, 1991). This showed directly that β-carotene acted as an antiphotooxidant rather than an antiautoxidant.

Kinetic studies showed that the antiphotooxidant mechanism of β-carotene was actually due to its singlet oxygen quenching ability and not its ability to quench the excited triplet photosensitiser (Foote and Denny, 1968; Lee and Min, 1988, Lee and Min, 1990; Jung and Min, 1991; Rahmani and Csallany, 1998). Farmilo and Wilkinson (1973) found that without doubt the quenching of singlet oxygen by β-carotene is due to electronic energy transfer, which resulted in the triplet state of β-carotene. It has also been found that there was no appreciable consumption of β-carotene in photooxidation experiments; in fact one molecule of β-carotene quenched up to 250 molecules of singlet oxygen in some experiments (Foote and Denny, 1968). Several researchers have suggested its action may actually be due to selective oxidation (Kiristakis and Dugan, 1985). Another theory is that in addition to its ability to quench oxygen, β-carotene may act even earlier, filtering out specific wavelengths of light before it even reaches photosensitisers (Clements et al., 1973; Fakourelis et al., 1987). Clements et al. (1973) demonstrated that β-carotene had this additional antiphotooxidant property by using β-carotene in oil to act as a light 'shield', filtering the wavelength of fluorescent light so that a second sample of pure oil containing no antiphotooxidant had decreased photooxidation (Figure 7).

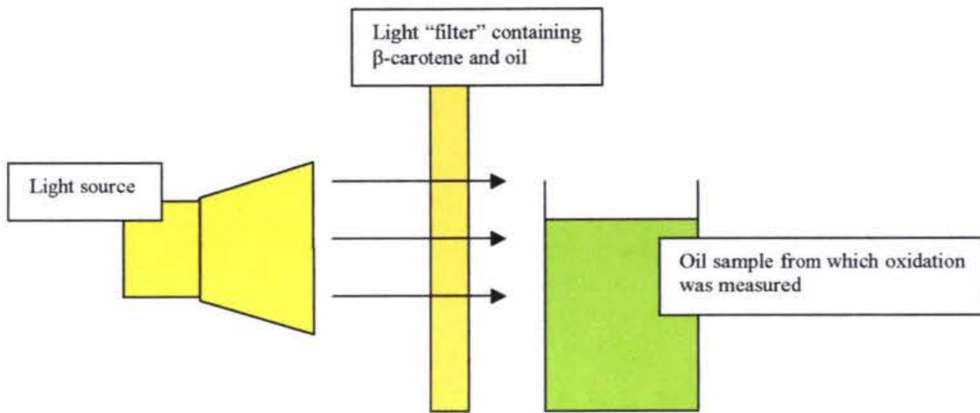
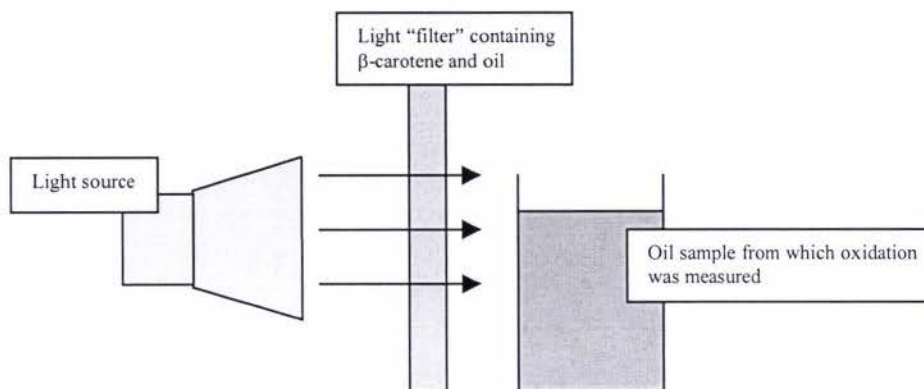


Figure 7. Light filtering protective effect of β-carotene as proposed by Clements et al. (1973) and Fakourelis et al. (1987)

The protection that β-carotene offers against photobleaching is the same mechanism by which carotenoids protect against photodynamic damage in living systems (Foote et al., 1970; Rawls and Van Santen, 1970). Photosynthetic organisms such as the plants from which edible oils are extracted are protected from the lethal effects of their own chlorophyll by carotenoids. This is readily shown by the rapid destruction of plant mutants that lack certain carotenoids (Foote and Denny, 1968). Thus, utilising β-carotene as an antiphotooxidant would seem a natural step to take in protecting lipids, whether it is in a biological system or in edible oils.

The antiphotooxidant nature of carotenoids is due to the conjugated structure of their carbon backbone. There has been much evidence to support this based on the increasing antiphotooxidative activities of carotenoids with increasing number of conjugated double bonds. Jung and Min (1991) showed that as the number of conjugated double bonds increased from 10 to 13, peroxide formation decreased significantly. That is, as an antiphotooxidant, canthaxanthin (13 conjugated bonds) was the most effective, β-carotene (11 conjugated bonds) the next most effective and β apo-8-carotenal (10 conjugated bonds) was the least effective of the three carotenoids tested. The conclusions of this study were reinforced by Lee and Min (1990) who found similar oxygen quenching rates between zeaxanthin, lycopene, isozeaxanthin and β-carotene – all carotenoids with 11 conjugated double bonds. The structures of these carotenoids are shown in Figure 8



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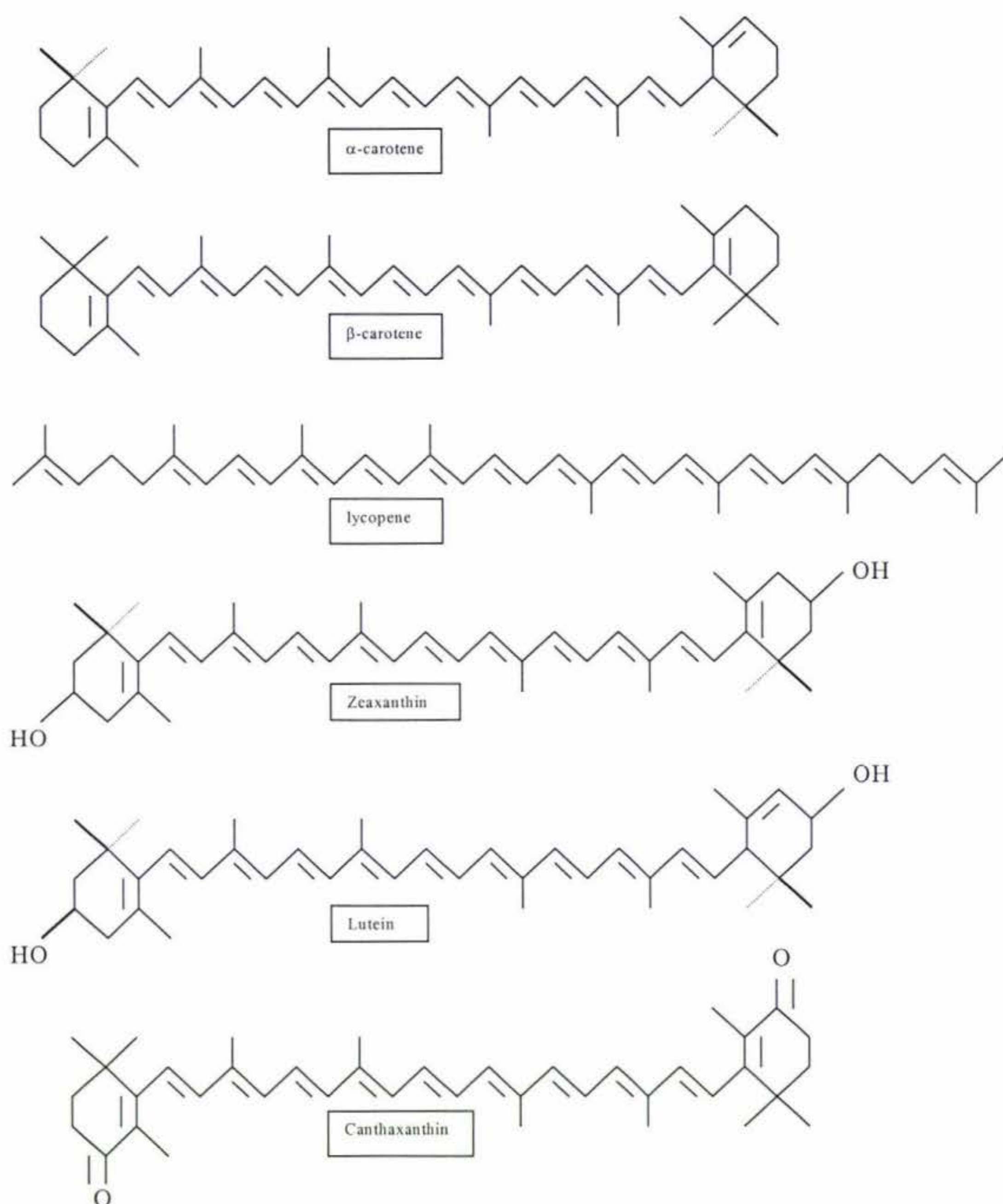

Figure 8. Carotenoids commonly found in the human diet (Deshpande et al., 1995)

From organoleptic testing it was shown that in stripped soybean oil, β-carotene at 5-10 ppm was acceptable but became unacceptable at 20 ppm (Warner and Frankel, 1987, Lee et al., 1997). This highlighted a practical limit for β-carotene in refined

edible oils. The same studies also showed that citric acid acted antagonistically with β-carotene. Beta-carotene was more effective in preventing the formation of volatiles in oils containing no citric acid compared to those that did have added citric acid (Lee et al., 1997). The opposite relationship (synergism) was observed when β-carotene was used in conjunction with α-tocopherol (Warner and Frankel, 1987). Warner and Frankel (1987) concluded that the tocopherols guarded β-carotene from free radical autoxidation, also α-tocopherol performed better in autoxidative situations when used in conjunction with β-carotene since it was protected from the effects of singlet oxygen.

2.4.4.2 Rosemary

Spices have been found to be effective antioxidants in a number of food products ranging from meat to confectionery. Of the spices, rosemary (*Rosmarinus officinalis*) and sage (*Salvia officianalis*) have been found to be the most effective. This may be attributed to specific components of rosemary, namely carnosol, a phenolic diterpene, as well as rosmaridiphenol, rosmarinic acid, carnosic acid, rosmanol, isorosmanol and epirosmanol (Inatani et al., 1983). However, isolation of these components from rosemary requires large quantities of solvent and plant material to obtain significant quantities of the pure antioxidant (Hall et al., 1994).

Dried rosemary leaves were added as an ingredient at 0.15% to a basic oil in water emulsion that simulated a dressing to test the efficacy of rosemary as an antioxidant against known standards (Madsen et al., 1998). The oxidative protection offered against autoxidation by rosemary during dark storage was found to be significantly better than 80 ppm PG which is the standard antioxidant for this type of product. The effectiveness of rosemary as an antioxidant was based on the development of conjugated dienes, peroxide value and headspace hexanal. The net antioxidative effect of rosemary was also maintained in a photooxidative system when the emulsion was exposed to 850 lux fluorescent light, suggesting activity as both an antiautoxidant and antiphotooxidant. The methanol extract of rosemary used at the same effective concentration was also tested in this research but was found to be less effective than the dried rosemary leaves.

This suggested the synergistic action of several antioxidant components of rosemary.

Of the extracts of rosemary, rosmariquinone has been the most well studied in terms of antioxidant ability. Rosmariquinone is an ortho-quinone diterpenoid found in rosemary and acts as a hydrogen donating antioxidant (Clifford et al., 1998). Thus, rosmariquinone can be used synergistically with primary antioxidants such as α-tocopherol by recharging them with hydrogen. It can also act synergistically with β-carotene. This was shown by Hall et al. (1994) who found that rosmariquinone had greater antioxidant activity in non-stripped soybean oil than in stripped soybean oil which contained no natural antioxidants. Weng and Gordon (1992) showed that rosmariquinone did not act as a metal chelating agent, since adding iron was very effective in reducing its antioxidant effects. Rosmariquinone can be produced synthetically but its use as an antioxidant in food will rely on the determination of all toxicological data (Hall et al., 1994). Clifford et al. (1998) found that rosmariquinone had antioxidant activity in both light induced oxidation and autoxidation. In the case of an autoxidised lard system, its antioxidant activity was found to be greater than BHA and other quinones when tested using a Rancimat at 100°C (Weng and Gordon, 1992; Hall et al., 1994).

Rosemary oleoresin is a mixture of monoacylglycerides and diacylglycerides extracted from rosemary oil in vegetable oil (Hall et al., 1994). It is sold as a flavouring with the added feature that it exhibits antioxidant activity (Hall and Cuppett, 1993). The antioxidant activity of rosemary oleoresin is less than that of rosmariquinone, because rosmariquinone is a relatively pure substance and there is little interference from other components as was the case with oleoresins (Hall et al., 1994). This interference is likely to come from residual chlorophyll remaining in the oleoresin (~4ppm) even though it is classed as "substantially free" of chlorophyll (Hall and Cuppett, 1993). The presence of chlorophyll in rosemary oleoresin causes poor antiphotooxidant activity in both stripped and non-stripped soybean oil. The chlorophyll present causes photosensitisation (Hall and Cuppett, 1993). Also as rosemary oleoresin contains both monoacylglycerides and diacylglycerides, it can act as an emulsifier, reducing the interfacial tension between the oil and air which increases contact to oxygen and

subsequent oxidation (Hall and Cuppett, 1993).

Bleached and unbleached rosemary oleoresin showed maximal antioxidant activity in stripped and non-stripped soybean oil at 0.02% w/w and 0.05% w/w but a TBHQ antioxidant treatment had a significantly greater effect (Hall and Cuppett, 1993). Hall and Cuppett (1993) also suggested that the oleoresin acted similarly to TBHQ by acting as a free radical acceptor in autoxidation but to a lesser degree. Cuvelier et al. (1994) found that rosemary oleoresin was 3-7 times less active than BHT but this was probably due to the instability of some oleoresin components at the Rancimat test temperature of 110°C.

Several other studies have used 'rosemary extract', where antioxidant components of rosemary have been extracted using organic solvents. However, this application is less commercially viable than dried rosemary, rosmariquinone or rosemary oleoresin since isolation of these antioxidants requires large amounts of solvents and plant material to obtain significant quantities of pure antioxidant. Chang et al. (1977) found that from 100 g of dried rosemary leaves, 26 g of crude antioxidant could be extracted. Purification then lowered this yield down to 10 g, which was 10 % of the weight of the original dried, powdered rosemary leaves. Chang et al. (1977) and Chen et al. (1992) also favoured more polar solvents, specifically methanol for this process as it increased the extraction yield. It was found that this extract had the same effectiveness as a synergistic mixture of BHA, BHT, PG and citric acid in animal fat and an even greater effectiveness in vegetable oils in a Schaal oven accelerated oxidation test at 60°C (Chang et al., 1977). The addition of 0.05 % ascorbic acid increased the antiautoxidative efficacy of rosemary antioxidant even further in these systems due to synergistic activity. The same extract appeared to act as an antiphotooxidant as well, where it improved the flavour stability of soybean oil held under diffuse daylight.

The antioxidant activity of rosemary extract depends mainly on their content of carnosic acid and carnosol (Chen et al., 1992). Each of these compounds are present at about 0.35 % in dried rosemary leaves (Munne-Bosch et al., 2000). However, there may be a strong variation in the content of carnosic acid and other phenolic diterpenes in

rosemary during growth that may increase in response to water stress and decrease in response to light stress (Munne-Bosch et al., 2000). This suggests that they are in fact used to protect the plant from photooxidation.

Further fractionation of rosemary extract by HPLC has yielded a fraction with outstanding antioxidant ability (Chang et al., 1977). Some of these components, identified as rosmanol, epirosmanol and isorosmanol have shown high activity in lard and linoleic acid and were about four times more active than BHA or BHT (Inatani et al., 1983; Nakatani and Inatani, 1984). Another benefit of this isolation process was that the resulting antioxidants were both odourless and tasteless (Inatani et al., 1983)

2.5 Oxidation rate measuring techniques

Since oxidation is the primary cause of oil degradation, most accelerated shelf life tests are designed to expedite the oxidation process. The progress of degradation can be monitored by measuring either the disappearance of oxygen, or measuring the amount of oxidised products generated. To measure the oxidative history of an oil, measurements must be taken over time. These measurements may be made with any number of static tests.

2.5.1 Static oxidation tests

2.5.1.1 Peroxide value (PV)

Measuring the peroxide value (PV) of an oil either iodometrically or spectrophotometrically is by far the most commonly used method for measuring the rate of oxidation in lipids, especially for photooxidation (Endo et al., 1984a; Usuki et al., 1984; Kiristakis and Dugan, 1985, Jung and Min, 1991; Lee and Min, 1991; Jung and Min, 1992; Gutierrez-Rosales et al., 1992; Hall and Cuppett, 1993; Rahmani and Csallany, 1998). This is because hydroperoxides are the primary identifiable intermediate in both autoxidation and photooxidation. Measurement of PV for extensive autoxidation is less purposeful as these peroxides are broken down as a result of

autolysis, but for photooxidation and the early stages of autoxidation it is an excellent indicator of the oxidative state of an oil and correlates very well with other tests (Lee and Min., 1991; Rossel, 1994).

When determining PV iodometrically, care must be taken to remove as much dissolved oxygen in the sample as possible as this can lead to incorrect high PVs due to the 'oxygen error' (AOCS, 1993). It has also been established that other possible sources of error in the iodometric methods include variation in the weight of sample, the type and grade of solvent used, variation in the reaction conditions such as time and temperature and the constitution and reactivity of the peroxides being titrated (Gray, 1978). The American Oil Chemists' Society iodometric peroxide value determination method (Cd 8-53) is highly empirical and any variation in procedure may affect results, thus adhering to a strict procedure for this test is very important (AOCS, 1993).

2.5.1.2 Conjugated diene products

The spectrophotometric measurement of conjugated dienes (CD) at 232nm is also a useful technique for measuring primary oxidation products and is similar to peroxide value determination (Eskin and Grossman, 1977; AOCS, 1993; Georgalaki et al., 1998. Since the CD value depends on the fatty acid composition of an oil, it cannot be compared from one species of oil to another easily (White, 1995). Like hydroperoxides, CD values tend to plateau when their breakdown presumably equals formation. The magnitude of the change in conjugated dienes is not readily related to the degree of oxidation, however the change can be used as a relative measure of oxidation (Gray, 1978).

Secondary oxidation products, including aldehydes and ketones can also be measured by analysing the absorbance at 270nm using a spectrophotometer (AOCS, 1993; Kiritsakis, 1998; Reed et al., 2001).

Comparison of these two measurements, called the conjugated diene ratio, can give an indication of non-conjugated peroxides formed. Since non-conjugated peroxides are

specific to lipids that have been oxidised by singlet oxygen, these two measurements can allow the identification and measurement of photooxidation more precisely.

2.5.1.3 Headspace oxygen depletion

Headspace oxygen depletion is a direct indication of the amount of oxygen consumed during storage of a food product and is entirely due to oxidation (Jung et al., 1991). Headspace oxygen depletion is measured by gas chromatographic analysis and has been shown to have an excellent correlation with peroxide value (R^2=0.99). It can be effectively used to study quenching mechanisms or kinetics of antiphotooxidants in the photosensitised oxidation of vegetable oil (Jung and Min, 1990; Jung et al., 1991; Jung and Min, 1992).

2.5.1.4 Thiobarbituric acid test

The thiobarbituric acid (TBA) method is based on the colour reaction between TBA and oxidation products of polyunsaturated lipids. TBA values may overestimate the extent of oxidation since other components, such as browning reaction products and protein and sugar degradation products may interfere with the formation of the TBA colour complex (Frankel, 1993). This method was used to monitor various forms of oxidation in olive oil and was shown to be more affected by temperature related reactions (autoxidation) than photooxidation. This is because photooxidation only produces primary oxidation products which the TBA test does not measure (Rossel, 1994).

2.5.1.5 Sensory testing

Sensory testing utilising descriptive attributes can also be used where trained panels can evaluate the odour and flavour of the oil. The most useful information is that related to the consumer acceptance of the product. Standard sensory techniques for the evaluation of rancidity in different oil types are available (AOCS, 1993; Kiritsakis, 1998).

2.5.1.6 Gas chromatographic (GC) analysis

Gas chromatographic techniques can be used to identify and quantify the presence of volatiles in the headspace of an oil sample. It may be used to determine total volatiles, which relates to the oxidative status of the oil, or to identify singular components, which relate to the flavour and odour of the oil.

Frankel (1993) ranked several static oxidation tests based on their accuracy in predicting stability, shelf life and consumer acceptability of food products, especially oil. This was in the following order of decreasing usefulness: Sensory (due to information most relevant to acceptability) > Headspace volatiles (closely related to sensory panel results) > Oxygen absorption (limited sensitivity but very relevant to oxidation) > PV (less sensitive and limited to lower temperatures due to hydroperoxide breakdown) > TBA (non-specific and unsuitable for oleic/linoleic acids).

2.5.2 Dynamic oxidation tests

The dynamic methods of oxidation measurement are generally much more involved than the static methods since they rely on performing at least one type of static test several times at defined intervals over a period of time for each sample. Data from this type of testing will show the oxidative history of oil and an oxidative 'future' may be predicted, depending on the scope of the test. This type of testing is very useful in determining the shelf life of oil or comparing types of oils or oil additives (such as antioxidants and prooxidants) against each other, providing a measure of resistance to oxidative rancidity (Rossel, 1994).

Although testing oil stability under ambient conditions may approximate real conditions of oil storage, the procedure is too slow to be of practical value. Slow oxidation can also make the system more sensitive to uncontrollable variables (Frankel, 1993). Sifi et al. (2001) showed that six months was not long enough to evaluate in full the oxidative stability of EVOO based on PV, conjugated diene products, tocopherol, fatty acid and

phenol level tests. In fact, extra virgin olive oil stored for 2 years in dark conditions at ambient temperature had almost no increase in peroxide value over that time (Kiristakis and Dugan, 1984).

Since the oxidative stability of an oil is in part a representation of the antioxidants present in the oil, oil tested should be stripped of tocopherols and other natural antioxidants if fundamental oxidative stability data is to be gathered (Frankel, 1993). This is not however a stipulation for practical experimentation, although a variance will be present due to naturally varying levels of antioxidants and prooxidants.

The most important consideration when attempting to conduct an accelerated oxidation test is that the conditions used should be as close as possible to the conditions under which the oil is stored (Frankel, 1993; Sifi et al., 2001). In addition, novel antioxidants should be tested against reference standards such as same molar concentrations of α-tocopherol, BHA or BHT (Frankel, 1993). Ragnarsson and Labuza (1977) concluded that each accelerated shelf life testing method must be calibrated for each individual oil formulation or makeup.

2.5.2.1 Induction period

The quantity of hydroperoxides formed during the photooxidation of unsaturated fatty acid methyl esters is directly proportional to the amount of light observed. Whereas for the autoxidation process the peroxide value is a function of both the amount of hydroperoxides initially present and time (Rahmani and Csallany, 1998). Thus when the oxidation of an oil was followed experimentally by measuring the amount of oxygen absorbed or the peroxide value, it was found that the course of oxidation shows two distinct phases. In the first phase (induction phase), oxidation progresses slowly and at a uniform rate, characterised by its linearity. This can usually be attributed to singlet oxygen oxidation, which is constant with time, all other things being equal. After the oxidation has proceeded to a certain point, the reaction enters a second phase which has a rapidly accelerating rate of oxidation (Hamilton, 1994). The second part of the curve is characterised by the autocatalytic production of hydroperoxides. The induction phase

is generally used as an indication of antioxidant effectiveness, and can be related to shelf life in food products (Frankel, 1993). The induction phases for three fabricated oil samples are shown in Figure 9.

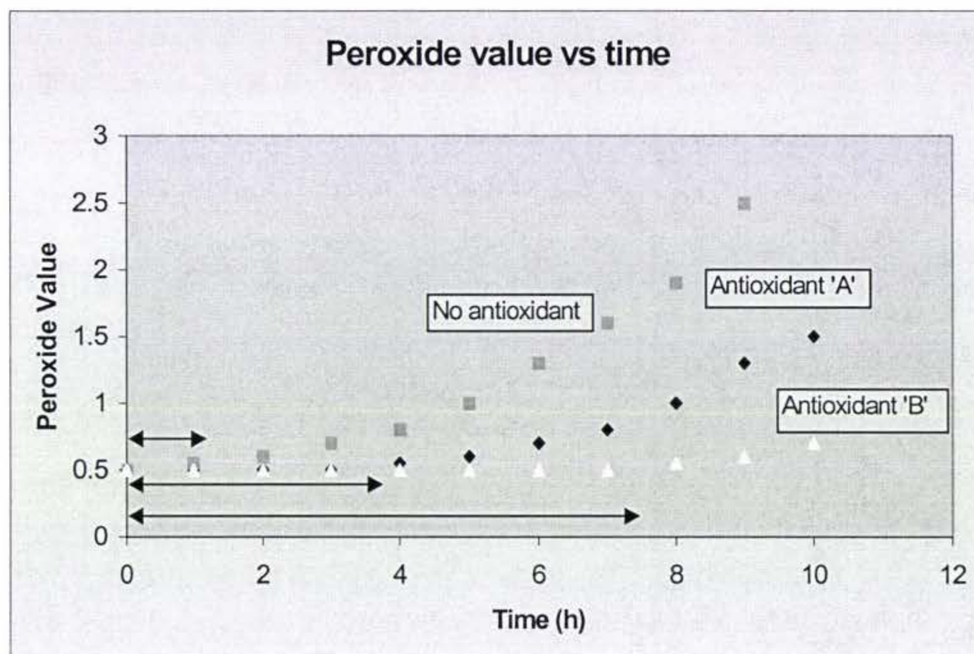

Figure 9. Example of PV vs. time curve showing effect of antioxidant 'A' and 'B' on induction time. Double headed arrows show induction period for each sample type (Hamilton, 1994).

2.5.2.2 Heating

Heating is the most common and effective means for accelerating oxidation (Frankel, 1993). It has been found though that the mechanism of lipid oxidation changes at high temperatures making high temperature analyses (>100°C) less relevant to actual storage conditions at ambient temperature. This can be a result of thermal decomposition of antioxidants, especially BHA, BHT and phenolic antioxidants (Frankel, 1993). Peroxide decomposition and an increase in oxygen solubility also occur at high temperatures (Ragnarsson and Labuza, 1977; Chen et al., 1992; Frankel, 1993). Moderate temperatures that are somewhat closer to ambient, such as those used in the Schaal oven test, may be used to minimise these unwanted side reactions

Ragnarsson and Labuza (1977) found that temperature accelerated test procedures were generally more accurate for vegetable oils and stabilised animal fats.

Schaal oven test

For the Schaal oven test, oil is placed in petri dishes and kept at a constant oven temperature (50-65°C). These samples are removed periodically for PV determinations and evaluation by a trained sensory panel (Warner, 1995). These moderate temperatures allow the Schaal test to avoid the breakdown of hydroperoxides and antioxidants at around 100°C and minimise breakdown of other components important to oxidative stability. Results for the oxidative stability based on PV at 60°C were in agreement with sensory evaluations and analyses of volatiles by gas chromatography (Frankel, 1993). The Schaal oven test is simple to perform, requires no special equipment, but requires a relatively large sample (~50g). Frankel (1993) found the Schaal oven test to have the fewest problems of all standard accelerated oxidation tests overall.

Active oxygen method (AOM)

The active oxygen method (AOM) was designed to measure the peroxides formed by reaction of active oxygen with oil (AOCS, 1993). It is one of the most commonly used stability test for oils and is an official American Oil Chemists' Society (AOCS) method (AOCS, 1993; Wan, 1995). For this test, 20 ml of oil is continuously aerated in a glass tube at 97.8°C and measured for PV periodically. The number of hours required for the oil to reach a PV of 100 mEq/kg oil is reported as the AOM time for the oil. This method is time consuming because it requires many PV titrations. The high temperature can also invoke undesired unknown oxidation mechanisms. Rancimat analysis has been considered an automated successor to this well known test due to its relative ease of operation and excellent correlation with AOM results (AOCS, 1993; Rossel, 1994).

Rancimat oxidative stability index (OSI)

The oxidative stability index (OSI) of olive oils has been tested successfully

using a Rancimat apparatus (Zhang et al., 1990; Gutierrez-Rosales et al., 1992). This device monitors the volatile compounds generated from an oil sample heated under constant aeration. Thermal conductivity data is then plotted against time to give an oxidation curve. Instead of measuring the time taken to reach a set amount of peroxides, the Rancimat measures the induction period. This is an excellent indicator of shelf life stability and antioxidant efficacy (Frankel, 1993). However the method of detection, conductivity of volatiles formed and trapped in water, may be sensitive to breakdown products other than peroxides. Thus, it is advisable to compare the relative stability of oils at various temperatures. Rancimat analysis also requires a high level of oxidation for measurable results and an unreliable endpoint (Frankel, 1993). However, Wan (1995) concluded that Rancimat testing offers an efficient and rapid prediction of oxidative stability.

2.5.2.3 Light reactors

Light reactors and photosensitisers can be used to accelerate photooxidation similarly to heat accelerating autoxidation (Lee and Min, 1991). For photooxidation testing, oil samples are held in a clear vessel at ambient temperature and are exposed to a well-defined light source (Chen et al., 1996). Measurements may be performed by a trained sensory panel by monitoring 'grassy', 'reversion' or 'light-struck' flavours (Warner, 1995). Instrumental techniques such as oxygen uptake or PV determination are very suitable. PV determination is particularly suitable since only primary oxidation products are formed from photooxidation (Hahm and Min, 1995).

2.6 Chlorophyll measurement

2.6.1 Spectrophotometric measurement

The American Oil Chemists' Society (AOCS) official method Ch 4-91 for determining the chlorophyll-related pigments in oil relies on the measurement of light absorbance at 670nm relative to baseline measurements at 630nm and 610nm (AOCS, 1993). It is applicable for all oil types except for those that are deodorised or hydrogenated.

Measurement at 670nm using this method measures predominantly pheophytin *a* but gives in indication of total chlorophyll pigments present (AOCS, 1993).

The spectrophotometric method of chlorophyll determination has also been used for research into the breakdown of chlorophyll in food products other than oil (Vernon, 1960; Venning et al., 1989; Ward et al., 1992; Heaton et al., 1996; Weemaes et al., 1999). These studies measured different absorption wavelengths for the determination, namely the absorption maxima near 665nm for chlorophyll *a*, *b* and their breakdown products using absorptivity at 625.5nm and 705.5nm as a baseline (Ward et al., 1992). These methods involved the extraction and determination of the chlorophyll using acetone as a solvent rather than determination in the matrix.

2.6.2 Colorimetric measurement

Colorimeter devices have been successfully used to measure the degradation of chlorophyll in several chlorophyll containing food products including broccoli juice and peas (Weemaes et al., 1981; Steet and Tong, 1996). Since the negative *a* value of the Hunter Lab colour scale reflects the greenness of a sample, this colour parameter was selected as an indicator of green colour loss (Weemaes et al., 1999; Steet and Tong, 1996). It has been reported to be linearly related to both green colour and consumer acceptance in peas (Steet and Tong, 1996). The change in Hunter *b* value during the chlorophyll degradation of broccoli juice was very limited (Weemaes et al., 1999).

The negative *a* value approached an asymptotic value of 6.0 independent of reaction temperature during chlorophyll degradation in peas (Steet and Tong, 1996). When the asymptotic value was reached, there was no remaining chlorophyll *a* or *b* in the mixture as determined by HPLC analysis. The kinetic parameters for loss of green colour as measured by the Hunter *a* value fell between those of chlorophyll *a* and *b* suggesting that green colour loss was a consequence of losing both chlorophyll *a* and *b* (Steet and Tong, 1996)

Weemaes et al. (1981) found that from the point of view of consumers, green colour as

measured objectively by the Hunter *a* value was more important than residual chlorophyll content.

2.6.3 High pressure liquid chromatography

Total chlorophyll content can be accurately measured by its components (chlorophyll *a* and *b*; pheophytin *a* and *b*) using high pressure liquid chromatography (HPLC). This is the most accurate method for determining the components of chlorophyll and has been widely used in chlorophyll degradation studies in various food products (Steet and Tong, 1996; De le Cruz-Garcia et al., 1997; Yamauchi et al., 1997; Ryan-Stoneham and Tong, 2000). Measurement of chlorophyll content by HPLC analysis first involves extraction of the chlorophyll from the substrate using acetone as a solvent. This extract is then injected onto an HPLC column, followed by measurement of absorption peaks using a UV-visible spectrophotometer set at 665nm or 658nm (Steet and Tong, 1996; Yamauchi et al., 1997; Ryan-Stoneham and Tong, 2000). The peaks obtained from these elutions can be calibrated with standards of pheophytin *a* and *b* or chlorophyll *a* and *b* for quantification of chlorophyll pigment concentrations

2.7 Effect of packaging materials on oil quality

The effects of packaging and storage materials on virgin olive oil have been studied by Kiristakis and Dugan (1984). They found that the peroxide value of virgin olive oils stored in dark conditions in closed tins or glass containers was small due to the limited amount of oxygen in the headspace of the containers, even after a period of 2 years. However in (polyvinyl chloride) PVC containers, peroxide formation was much greater due to the probable intrusion of oxygen as a consequence of plastic permeability. It was also found that bottles that were covered with aluminium foil had much slower peroxide formation than those without aluminium foil when stored under direct sunlight or diffused store light. This was due to the decreased effect of photooxidation. It was concluded that olive oil should be stored in bottles which are not transparent to light or permeable to oxygen in order for oxidative deterioration to be minimised during storage (Kiristakis and Dugan, 1984).

3 Materials and Methods

3.1 Oil samples

3.1.1 Extra virgin avocado oil

Extra virgin avocado oil (EVAO) was supplied by Olivado New Zealand from avocado oil that was produced at their production plant in Kerikeri, New Zealand. It was produced using Alfa-Laval olive oil extraction equipment with parameters modified to suit avocado oil production. Following production, avocado oil was sparged with nitrogen and stored in large, stainless steel vessels. Samples used were dated by production date and tested for chlorophyll level and peroxide value before use. Since oil evaluation tests were run over a large time frame, different oil samples were used in different tests. Oil used for each test was dated as the number of months old at the time of testing.

3.1.2 Refined, bleached and deodorised avocado oil

Refined, bleached and deodorised (RBD) avocado oil was also supplied by Olivado New Zealand. The oil was refined at Bakels Edible Oils (New Zealand) from Olivado EAVO. The same oil sample was used for all tests (refined 17/05/01) and dated as the number of months since the RBD process at the time of testing. This sample had no detectable peroxides, free fatty acids or chlorophyll directly after refining.

3.2 Accelerated oxidation reactor

3.2.1 Compressed air and nitrogen

The compressed air and nitrogen used in the accelerated oxidation reactor were supplied by BOC Gases Ltd (New Zealand). The nitrogen grade was 'food fresh' and the air grade was 'dry air'.

3.2.2 Gas fittings, regulator and volumetric flow meter

The regulator used for nitrogen/air supply was the BOC 6000 200 kPa argon regulator from BOC Gases Ltd (New Zealand). The volumetric flow control meter used was a 0-25 $lmin^{-1}$ ball bearing meter from BOC Gases Ltd (New Zealand). All brass fittings and hoses were also supplied by BOC Gases Ltd (New Zealand).

3.2.3 Light source and light intensity meter

The light source for the reactor was from two 46 mm long 15 W Cool white fluorescent light bulbs (Osram Sylvania, USA). Light intensity at the sample was varied by turning on one or two bulbs at a time and also by varying the distance of the light source to the sample by using the reactor box collar. Luminosity (light intensity) was measured by the IM3 luminosity meter (Torcon Corporation, Japan).

3.2.4 Water bath and stainless steel jacketed vessels.

The water bath used to circulate heated water through the jacketed stainless steel vessel was supplied by Grant (England: Model Y28) and the stainless steel jacketed vessels were custom made by Mike Christie Sheet-metals Ltd (New Zealand). The internal dimensions of the stainless steel jacketed vessels were 100 mm diameter × 155 mm height with a water jacket thickness of 10mm.

3.2.5 Reactor box and collar

The reactor box and collar was custom made from 12 mm thick laminated multi-directional fibre (MDF) board. The internal dimensions of the box were 550 mm wide × 300 mm deep × 330 mm high. A collar was also made to vary the distance of the light source (which was mounted on the box lid) to the base of the box. When used, the collar added 150mm to the height of the box.

3.2.6 Extraction fan

A 80 mm × 80 mm × 25 mm 12 volt computer fan and 3-12 volt 300 mA power supply (Dick Smith Electronics Ltd., New Zealand) was used to extract moist air from the reactor box.

3.3 Hot air oven apparatus

3.3.1 Hot air oven

The oven used for hot air oven accelerated oxidation testing was a 2000W Contherm digital series model 250M (Contherm Scientific, New Zealand).

3.3.2 Moisture dishes

The moisture dishes used in the hot air oven tests were machined from aluminium and had an internal diameter of 65 mm and a height of 25 mm. Lids were of 69 mm diameter with a 1 mm lip and were not airtight.

3.4 Spectrophotometry equipment

3.4.1 Spectrophotometer and PC software

All spectrophotometric analyses in this research were performed using the Shimadzu UV-1601PC spectrophotometer (Shimadzu Corporation, Japan). The software used for analysis of spectrophotometric data was UVPC Personal Spectroscopy Software (Version 3.7) also supplied by Shimadzu Corporation (Japan).

3.4.2 Cuvettes

A pair of matched 1.0 cm path-length quartz cuvettes were used for spectrophotometric

analyses. These were supplied by Scientific Supplies Ltd. (New Zealand).

3.5 Peroxide value (PV) determinations

3.5.1 Solvent (isooctane)

Analytical grade 2,2,4 Trimethyl pentane (isooctane) (Labscan, Ireland) was used as the solvent for all PV determinations and as the solvent and blank for all spectrophotometric analyses.

3.5.2 Reagents

The following reagents were used for PV analyses: Analytical grade sodium thiosulphate (BDH Chemicals, England), analytical grade potassium iodide (BDH Chemicals, England), analytical grade soluble starch (BDH Chemicals, England) analytical grade glacial acetic acid (Scharlau, Spain).

3.6 Antioxidant evaluation

3.6.1 Antioxidants

Ascorbyl palmitate (E304) and mixed tocopherols (E306) were supplied by Roche (USA). Herbor 025 Rosemary oleoresin was supplied by Robertet (France). Citric acid monohydrate was analytical grade (May and Baker, England).

3.6.2 Solvent

Analytical grade absolute ethanol (Scharlau, Spain) was used to dissolve ascorbyl palmitate and citric acid before addition to the test oil.

3.7 Colorimetry

3.7.1 Colorimeter

The CR300 colorimeter (Minolta, Japan) was used for measuring the colour of avocado oil. A standard white tile supplied with the colorimeter (L = 97.79, a = -0.53, b = +2.28) was used for calibration prior to colour measurement.

3.7.2 Sample containment

Oil samples were held in 25mm diameter × 150mm high (~50ml) boiling tubes during accelerated shelf life trials. These were placed into the CR-A70 tube holder (Minolta, Japan) with the colorimeter connected for analysis.

3.8 Oil stability index (OSI) analysis

3.8.1 OSI apparatus

The apparatus used for OSI analysis was the model 679 Rancimat (Metrohm, Switzerland).

3.9 Methods

3.9.1 Development of the accelerated oxidation reactor

Cold-pressed avocado oil contains many minor constituents that are extracted from the fruit along with the oil. Among these components are various levels of oxidation promoters (e.g. chlorophyll) and oxidation inhibitors (e.g. tocopherols, carotenes). These components are expected to have a profound effect on the oxidative stability of the oil. Since there have been very few studies on the oxidation of avocado oil, especially crude (virgin) oil, it was of interest to examine the effects of elevated

temperature, light and oxygen levels on the oxidation of avocado oil. The information from this preliminary investigation was used to develop more specific and sensitive accelerated oxidation tests. An accelerated oxidation reactor was developed to perform this task.

3.9.1.1 Light supply

After careful consideration of the literature, fluorescent light was chosen as the light source. The same 15W cool white fluorescent tubes (Sylvania, USA) were chosen as those used in research performed by Lee and Min (1988) and Jung et al. (1991). This was because these lamps are common in supermarkets and factories where oil is stored, since their wavelengths closely simulate daylight (Osram Sylvania). The fluorescent lights used are shown attached to the reactor lid in Figure 10. Daylight was not used because of its variability.

To eliminate the effect of stray light, a box was built from laminated custom wood to house the reactor (Figure 11). The two lamps were placed on the inside of the box lid. A collar was made to fit in between the top of the box and the lid so that the lid could be raised to reduce light intensity to the oil samples sitting on the floor of the box. Two holes, each 30mm in diameter were made in the lid of the box directly above where each of the oil samples were held during experiments. These sample ports allowed oil samples to be removed by a micropipette without having to remove the lid from the reactor. The ports were covered with aluminium foil to block out ambient light during experimentation. Light intensity was measured with the luminosity meter as 4500 lux at the position of the oil sample, as used by Kiristakis and Dugan (1985). This intensity was suitable for the extent of oxidative deterioration encountered in the time period for each treatment of avocado oil. This time period was 7 hours, similar to that used in experiments performed by Jung et al. (1991). Light intensity with both lamps turned off was 0 lux, hence no stray light penetrated the reactor box.

Two levels were chosen for light intensity:

1. 4500 lux, used for promoting photooxidation
2. 0 lux, used for eliminating photooxidation

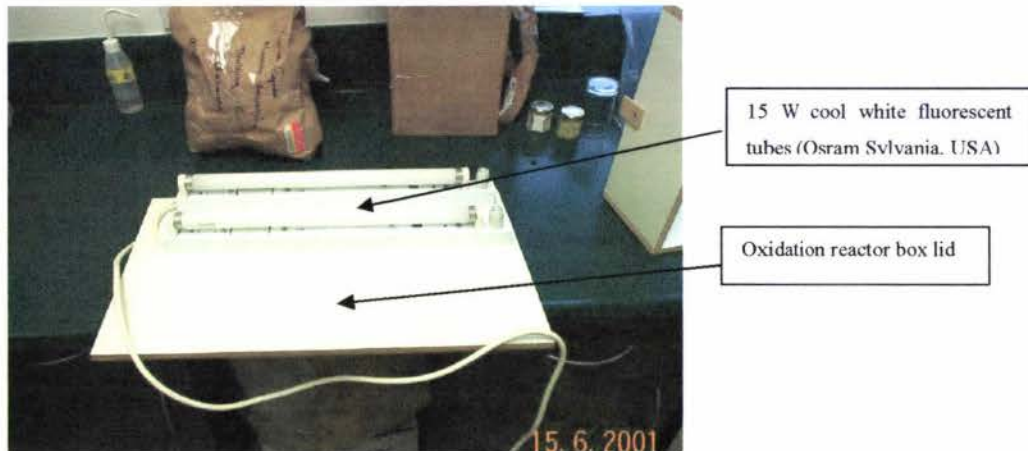

Figure 10. Oxidation reactor lid and light supply

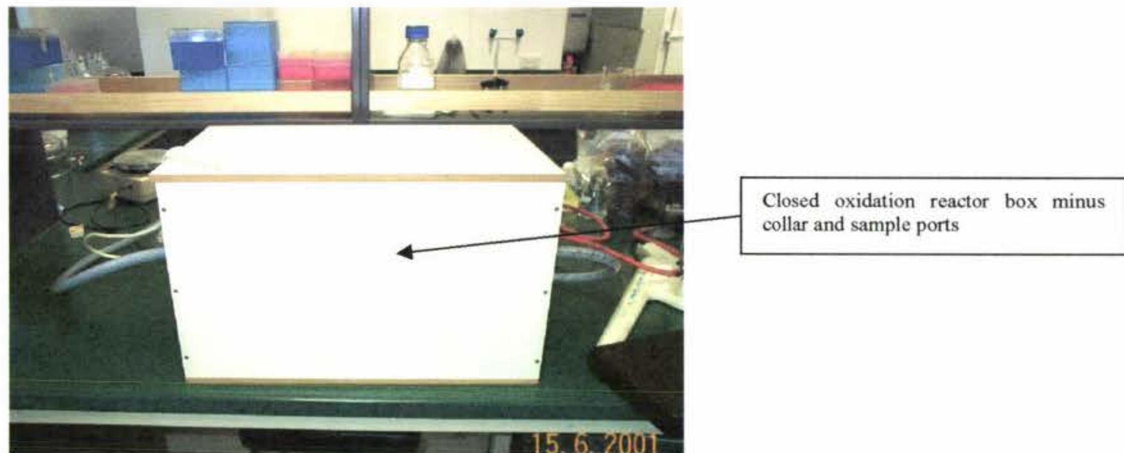

Figure 11. Closed oxidation box

3.9.1.2 Heating of the oil sample

Two stainless steel jacketed vessels were used as controlled temperature water baths, into which the 50ml pear shaped flasks holding the oil samples were suspended. Water pumped into the stainless steel jacketed vessels was heated in an external water bath

(Grant, England). This allowed the oil to be heated with minimal obstruction to the light source. This temperature maintenance system is shown in Figure 12 and Figure 13.

Two levels of heating were used:

1. 25°C. This temperature closest to ambient that could be achieved with stability using the water bath.
2. 60°C. This was the maximum temperature that could be used for accelerating autoxidation using the same reaction mechanism as at ambient whilst minimising side reactions and breakdown of antioxidants (Werman and Neeman, 1986a; Werman and Neeman, 1986b; Frankel,1993)

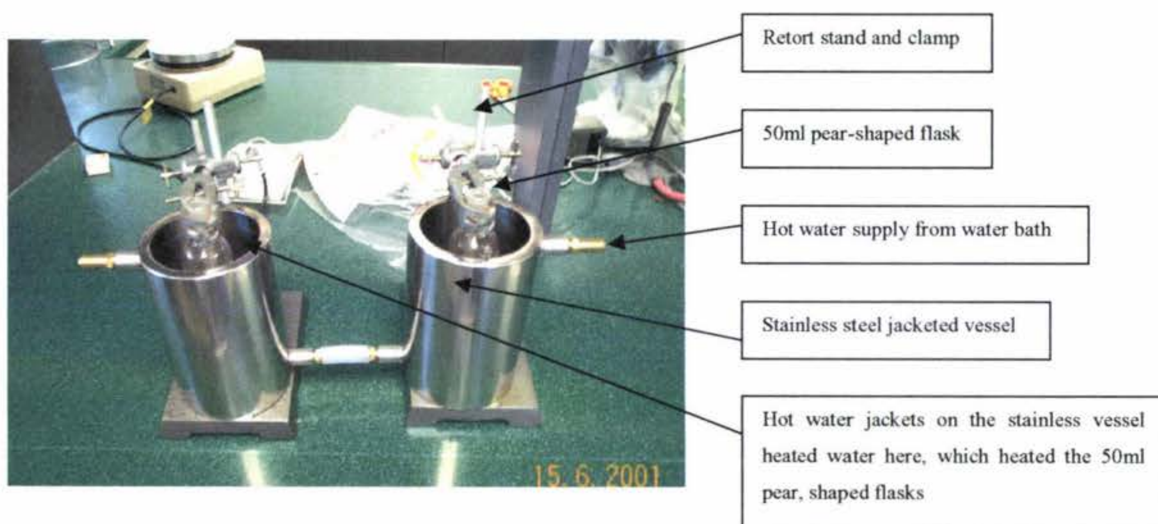

Figure 12. Temperature maintenance system for oxidation reactor

3.9.1.3 Gas supply

Nitrogen and oxygen were bubbled through oil samples via a semi-rigid 1mm internal diameter nylon hose that extended to the bottom of the pear shaped flask (Figure 13). Gas supply for this apparatus was supplied by food-grade compressed nitrogen gas or dry air. The flow control meter was set to 1 $lmin^{-1}$, which was split into approximately 0.5 $lmin^{-1}$ for each sample during experimentation. This was assumed sufficient to saturate the oil sample with the gas in use. Three levels of oxygen concentration were chosen for this factor:

1. 0 % oxygen. Nitrogen gas only bubbled through since nitrogen bubbles would

displace any dissolved oxygen.

2. 21 % oxygen (atmospheric level). No gases bubbled though, dissolved oxygen concentration assumed to be at equilibrium with atmospheric oxygen.
3. 100 % oxygen when compressed air was bubbled through. This was taken as 100% since any oxidation reaction would have access to excess oxygen. Pure oxygen was not used due to limitation on regulators and flow meters, and the use of pure oxygen can lead to a fire risk.

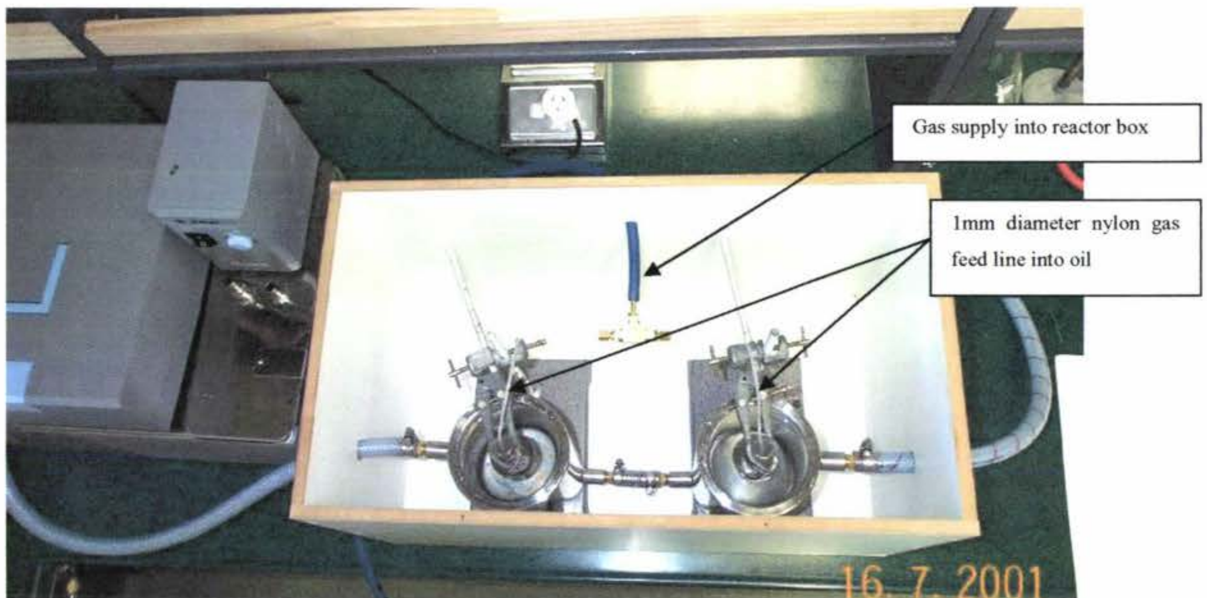

Figure 13. Gas supply into oxidation reactor box and oil samples

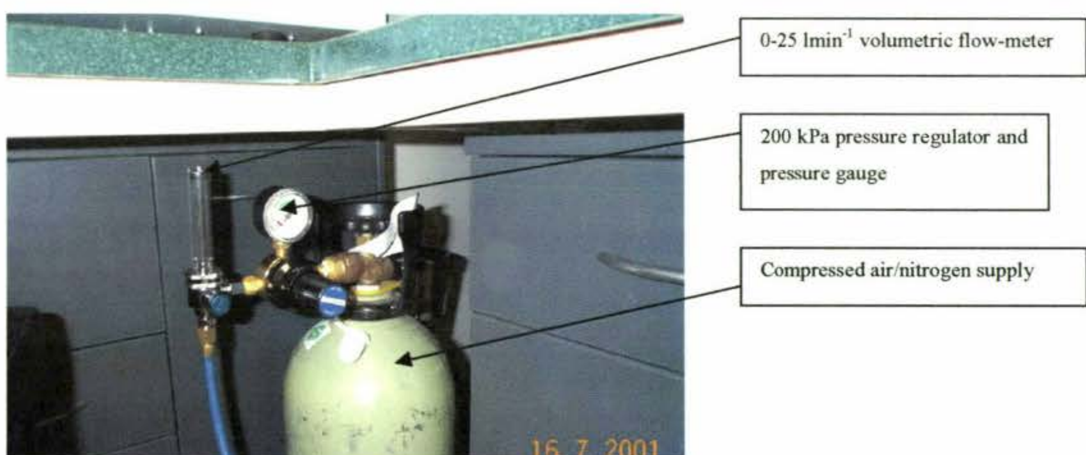

Figure 14. Gas supply for oxidation reactor

A lot of moisture was generated from the water in the stainless steel vessels during operation of the reactor at 60°C. An extraction fan was installed in the box just above the gas feed line. A 12 V computer fan with a low current isolated power supply was chosen for this task to avoid potential electrocution. A downward facing, black, 90° plastic elbow was placed over the fan to stop stray light entering the box.

A schematic diagram of the accelerated oxidation reactor as developed is shown below in Figure 15. A photograph of the finished reactor is also shown in Figure 13.

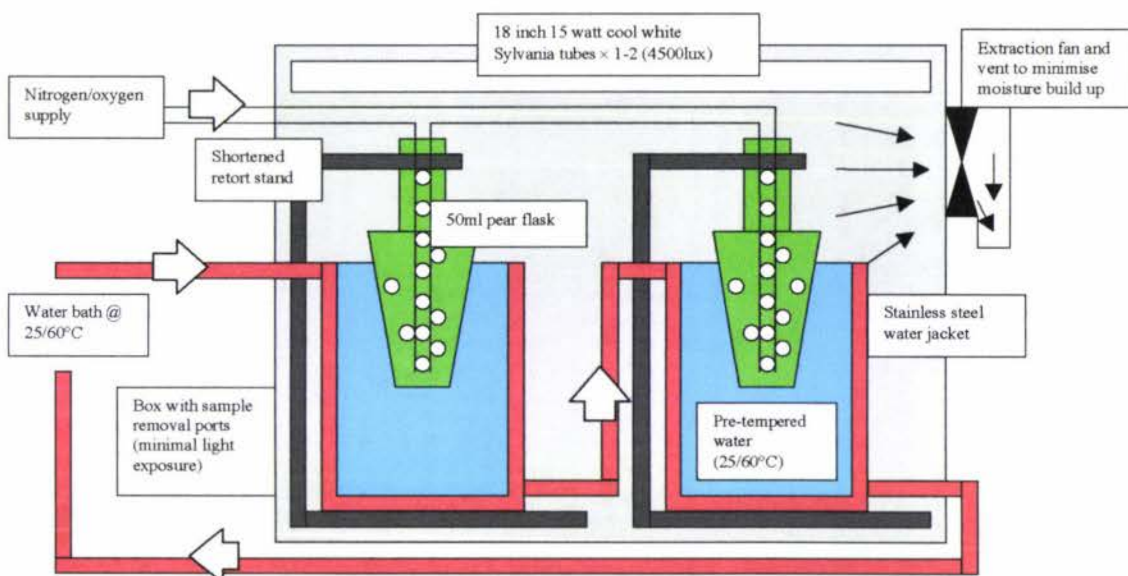

Figure 15. Schematic diagram of accelerated oxidation reactor

3.10 Operating Procedure for Accelerated Oxidation Reactor

3.10.1 Start Up

After the water bath was topped up to its maximum level and water supply connections were checked, the water bath was set to the temperature according to the treatment described in the design matrix (Table 4) and allowed to heat up. After the set temperature was achieved, 50 ml EVAO was added to each of the two 50ml pear shaped

shaped flasks for duplicate samples. This was done with laboratory lights turned off so that no stray light would affect the stability of the oil outside the experiment. The EVAO used for this series of tests was 3 months old at the time of testing. It had a mean initial PV of 0.96 ± 0.03 meq/kg oil and initial chlorophyll pigment content of 16.2 ± 0.1 ppm.

The pear-shaped flasks were held in the centre of the stainless steel heated vessels by shortened retort stands and flask clamps. The water level in the heated stainless steel vessels was bought up to that of the oil in the flask to ensure correct heating. Nylon gas supply tubes were placed into the bottom of both flasks and the appropriate supply according to the design matrix (Table 4) was turned on at the cylinder. Flow rate from the gas cylinders was set at $1 l min^{-1}$ and an equal flow rate to each flask was ensured. At this stage the reactor looked like the picture in Figure 13.

Once preparation was complete, the lid was placed onto the box and the sample ports were covered with aluminium foil. The light source was then turned on according to the design matrix and the time counter was started. The extraction fan was also switched on to eliminate excessive moisture build-up, especially for temperature treatments of 60°C. For these treatments, the water level in the jacketed vessels was topped up hourly to replace water lost due to evaporation.

Treatments were chosen according to an unblocked randomised factorial design matrix as created with MINITAB statistical software (shown in Table 4).

Table 4. Design matrix for experimental treatments in accelerated oxidation reactor

Random Order	Light level	Temperature	Oxygen Level
8	4500 lux	25°C	0%
10	4500 lux	25°C	21%
1	4500 lux	25°C	100%
5	4500 lux	60°C	0%
7	4500 lux	60°C	21%
9	4500 lux	60°C	100%
6	0 lux	25°C	0%
3	0 lux	25°C	21%
2	0 lux	25°C	100%
11	0 lux	60°C	0%
4	0 lux	60°C	21%
12	0 lux	60°C	100%

3.10.2 Sampling

Laboratory lights were turned off to minimise the effect of stray light during sampling. Foil covering the sample ports was then folded back and a 1000 μl micropipette was used to remove a suitable amount of oil directly into the test vessels for PV determination and cuvettes for chlorophyll pigment determination. This sampling was performed once for each of the two flasks. Once sample collection was complete, foil hatches were closed again.

3.10.3 Testing

PV and chlorophyll pigment determinations were performed once for each flask, giving duplicate readings for both chlorophyll and PV for each treatment. These tests were performed every hour for 7 hours for each treatment.

Results were analysed by ANOVA for single effects and interactions using MINITAB statistical software.

3.11 Method for Determining Peroxide Value (PV) in Avocado Oil

3.11.1 Modifications made to standard AOCS Cd 8b-90 method

The volume of oil used for each accelerated oxidation test in this research was small; approximately 50ml. Since PV determination is destructive to the oil, there was a limited quantity of the oil available for periodic analysis. The American Oil Chemists' Society (AOCS) method for iodometric determination requires 5 g of oil sample (AOCS, 1993). This was modified to 2 g for use in this research so as not to exhaust sample oil supply.

Since the amount of oil sample used for PV analysis was decreased, the amount of solvent used (3:2 acetic acid/isooctane mix) was also decreased, from 30ml to 20ml. The solvent level was not decreased in the same proportion as the oil because there are high levels of chlorophyll in EVAO, making the colour change titration endpoint harder to recognise. The greater dilution with solvent made the titration endpoint colour change easier to distinguish.

The concentration of the titration reagent, sodium thiosulphate, was decreased 5 fold from 0.01 mol l^{-1} as described in the AOCS method, to 0.002 mol l^{-1}. This was to match the decrease in oil tested and for the generally low levels of peroxides that were measured in this research; between 0 and 15 meq peroxides/kg oil. The equation for the calculation of PV was subsequently modified for the new parameters and simplified.

3.11.2 Reagents

0.002mol l^{-1} sodium thiosulphate solution. Sodium thiosulphate solution ($Na_2S_2O_3$) was made at 0.1 mol l^{-1} and standardised using the method in the AOCS official method Cd 8b-90 (AOCS, 1993). This solution was diluted daily to 0.002 mol l^{-1} by adding 2 ml

of the 0.1 mol l^{-1} solution to 98 ml of distilled water in a 100 ml volumetric flask. The 0.1 mol l^{-1} solution of sodium thiosulphate was made monthly and stored away from the light when not in use.

Saturated potassium iodide solution. This was made by adding 10 g of potassium iodide crystals to approximately 5 ml of distilled water followed by thorough stirring. Before using, undissolved crystals were checked for in the solution to ensure that the solution was saturated. Saturated potassium iodide solution was made daily for PV determinations to ensure there had been no conversion from iodide to iodine that could upset results. When not in use, the potassium iodide solution was stored in the dark.

Starch solution. For the 1 % starch indicator solution, 1 g of unmodified soluble starch was added to approximately 2 g of cold, distilled water. These were mixed together to form a slurry. Boiled distilled water (200 ml) was then added to the slurry and boiled for a further 2 minutes. The starch solution was kept until the endpoint of titration from blue to colourless failed to be sharp, which was usually about 2-3 weeks.

3.11.3 PV determination

2 g of oil was accurately weighed into a 250 ml Erlenmeyer flask, followed by 8 ml isooctane and 12 ml glacial acetic acid. To this, 2 drops of the saturated potassium iodide solution was combined then swirled and placed in a dark cupboard for exactly 1 minute. After removing the mixture from the cupboard, 1 ml of the 1 % starch indicator solution and 30ml of distilled water was mixed into the solution to develop a blue-black colour.

Sufficient 0.002 mol l^{-1} sodium thiosulphate solution was then titrated into the mixture until the blue-black colour of the iodised starch indicator turn colourless. The endpoint of the titration was taken when the aqueous phase had no black, blue or brown colour remaining.

To ensure end-point was properly achieved, the sample was shaken vigorously prior to end-point of titration and the polar and non-polar phases were allowed to separate

for 30 seconds. This ensured that all peroxide had reacted with the iodine present.

Blank determinations using the isooctane solvent were performed daily to calibrate the test.

The peroxide value of the oil was determined by the following equation:

$$\text{PV (meq/kg oil)} = \frac{Na_2S_2O_3\text{titre} \times \text{molar concentration}[Na_2S_2O_3] \times 1000}{\text{sample weight (g)}}$$

3.12 Chlorophyll Determination

3.12.1 Modifications made to standard AOCS Ch 4-91 method

Cold pressed avocado oil contains significantly higher levels of chlorophyll than virgin olive oil (Werman and Neeman, 1986a; Gutierrez-Rosales et al., 1992). For this reason, avocado oil tested for chlorophyll pigments had to be diluted to stay in the effective absorbance range of the spectrophotometer. The ideal dilution for extra virgin avocado oil used in this research found by experimentation was 40% (v/v) - 2ml of oil was added to 3ml solvent.

The solvent chosen for this task was isooctane (Eyres, 2001). This was chosen because it was spectrally pure at the wavelengths tested and was much safer and less toxic than methylene chloride or methylisobutylketone as suggested by the AOCS official Ch 4-91 method (AOCS, 1993).

Quartz cuvettes with 10mm path-length were used instead of the 50mm path-length cuvette suggested due to the specifications of the spectrophotometer used.

The division constant (a function of the spectrophotometer) was set as 0.101 as used recently in virgin olive oil analysis by Reed et al. (2001)

3.12.2 Method

The UVPC software was set to make the spectrophotometer scan from 600-720 nm in the absorbance range of 0.0 – 2.0. Matched quartz cuvettes were cleaned thoroughly using detergent and water followed by moisture removal with acetone and drying in a dry air oven. After the UVPC software had indicated that the spectrophotometer was ready, the machine was calibrated with isooctane solvent by performing an auto-zero (blanking) run with both cuvettes clean and full of isooctane.

For testing the chlorophyll content of the oil, the sample oil was diluted to 40% in isooctane (v/v) by pipetting 3ml of isooctane in to a clean 5ml volumetric flask, then making up to 5ml by adding the oil sample. The flask containing the avocado oil solution was then stoppered and shaken vigorously for 10 seconds to ensure it was thoroughly mixed.

A clean and dry cuvette was rinsed with approximately 1 ml of the freshly shaken isooctane-oil sample. After the rinse was emptied, the cuvette was filled with the sample solution. The cuvette was then returned to the spectrophotometer sample port. The 600-720 nm scan was run and data was saved.

This procedure was repeated for each sample that required quantification of total chlorophyll pigments. Following each successful scan, the absorbance values at 710nm, 670nm and 630nm were recorded for analysis of total chlorophyll content.

Exposure of oil sample and sample in solution to light was minimised because of the photolabile nature of chlorophyll. Cuvettes were kept clean during sample testing.

Total chlorophyll pigment concentration was calculated by the following equation:

$$C_{670} = \frac{A_{670} - 0.5\left(A_{630} - A_{710}\right)}{0.101 \times L \times D}$$

Where:

C_{670} = Total chlorophyll pigment content (ppm)

A_n = Absorbance of oil sample at wavelength n

L = Path-length of cuvette (cm) = 1

D = Dilution = 0.4

3.13 Obtaining Hunter L a b values from extra virgin avocado oil

The Minolta CR300 colorimeter was calibrated using the standard white tile (L = 97.79, a = -0.53, b = +2.28) in calibration mode for each oil sample. Following calibration, the colorimeter head was attached to the liquid sample holder and fixed in place with the fixing bolt. The boiling tube containing 50ml of the oil sample was then placed in the liquid sample holder then tested using the Hunter Lab colour scale. *L*, *a* and *b* values were recorded for each sample.

3.14 Procedure for adding antioxidants to avocado oil

Since both ascorbyl palmitate and citric acid have limited solubility in oil, both were dissolved into absolute ethanol before being added to the oil sample. Half a gram of the ascorbyl palmitate (powder) or citric acid (granular) was placed in a 5 ml volumetric flask. This was made up to 5 ml with absolute ethanol. The flask was then stoppered, inverted and shaken several times to make sure all of the antioxidant was dissolved. When the ascorbyl palmitate could not be completely dissolved in the ethanol at room temperature, the mixture was heated indirectly in a warm water bath at approximately 50°C to ensure dissolution. The desired amount of antioxidant-in-ethanol solution was then removed from the flask by micropipette and made up to 100 ml with the oil sample in a 100 ml volumetric flask.

For rosemary oleoresin or mixed tocopherols, which were directly soluble in oil, 0.1 g of the antioxidant was added to 10 ml of the oil sample. The sample was then thoroughly mixed until the antioxidant was completely dissolved. 1 ml of this mixture was then transferred into a 100 ml volumetric flask and made up to 100 ml with the same oil. This gave a final concentration of 100 ppm of the antioxidant in the oil sample.

3.15 Hot air oven accelerated oxidation method

Before sample preparation began, moisture dishes used for holding oil samples were cleaned thoroughly with warm water and detergent then dried for 24 hours in a hot air oven at 60°C.

For the first hot air oven test, two moisture dishes were each filled with 50 ml of oil. One dish contained oil used for PV tests and the other dish contained oil used for chlorophyll pigment determination. Two 25 mm diameter × 150 mm high boiling tubes were also filled with 50 ml of the oil for analysis of Hunter Lab colorimetric values over the duration of the experiment. Since RBD avocado oil contained no detectable chlorophyll pigments and was essentially colourless, no samples were prepared for colorimetric analysis or chlorophyll pigment determination. Only one moisture dish was filled with RBD avocado oil and this was used for PV determination.

All sample preparation and testing was performed under low light conditions since photooxidation could seriously affect the accelerated autoxidation and PV, chlorophyll pigment and colorimetry results. Photooxidation was not considered in the hot air oven test.

Before placing samples in the hot air oven, all analyses were performed to provide zero time data for each oil sample.

All samples were covered with moisture dish lids then placed in the 60°C hot air oven, and the start time was recorded. Tests for each oil type were performed at regular intervals (approximately every 4 hours during daytime) to attain sufficient time-related data. This time was expressed as 'hours stored at 60°C'. Although moisture dish covers were removed during sampling, they were returned immediately afterwards and placed back in the hot air oven.

The EVAO samples used for these tests were 1 month old (PV = 2.1 ± 0.1 meq/kg oil;

chlorophyll pigment = 17.9 ± 0.3 ppm) and 9 month old (PV = 4.3 ± 0.2 meq/kg oil; chlorophyll pigment = 20.0 ± 0.4 ppm). RBD avocado oil was 4 months old at the time of testing and had a peroxide value of 5.7 ± 0.3 meq/kg oil.

This accelerated oxidation test was run until each oil reached a peroxide value of >10 meq/kg oil.

3.16 Q_{10} analysis and antioxidant trials

For these series of tests, the hot air oven was set to run continuously at 60°C.

After a set period (determined by sample type and specific experiment) duplicate oil samples were removed from the oven and sufficient oil from each container was removed to perform two PV and one chlorophyll determination. These three tests were performed for each duplicate. Aluminium lids were placed back on dishes as soon as the oil was removed, and returned to the hot air oven.

The base extra virgin avocado oils used for Q_{10} accelerated shelf life testing were 1 month old (PV = 2.2 ± 0.1 meq/kg oil; chlorophyll = 16.7 ± 0.3 ppm) and 10 months old (PV = 4.2 ± 0.1 meq/kg oil, chlorophyll = 19.42 ± 0.4 ppm). Refined, bleached and deodorised oil used for this test was 7 months old at the time of testing with a PV of 5.7 ± 0.2 meq/kg oil.

The base extra virgin avocado oil used for antioxidant evaluation was 4 months old at the time of testing with an initial peroxide value of 0.7 ± 0.1 meq/kg oil and chlorophyll pigment concentration of 16.2 ± 0.2 ppm.

A second oven was used for Q_{10} analysis that was set to 50°C. All other techniques shown above were also applied to this testing.

3.17 Oil stability index (Rancimat) analysis

Oil stability index analysis was used as a screening test for antioxidants that were considered effective in EVAO. This testing was performed using an automated Metrohm 679 Rancimat machine (Switzerland) according to the AOCS official method 12b-92 at 100°C (AOCS, 1993).

The Rancimat is an automated machine that can rapidly determine the oxidative stability of an oil by the oil stability index method; AOCS official method Cd 12b-92 (AOCS, 1993). It works by bubbling heated, purified air through an oil sample held in a water bath at 100°C and collecting the volatile oxidation components from the exiting air stream in distilled water. The volatile components increase the conductivity of the water as the oil becomes oxidised. The oil stability index is defined as the point of maximum change of the rate of oxidation.

Antioxidant screening tests from Rancimat analysis gave indicative antioxidant effectiveness in EVAO. This data was used to determine which antioxidants would be examined further at lower temperatures for greater sensitivity using the hot air oven test.

3.18 Avocado oil production line analysis and process improvement

A process flow diagram was constructed based on the avocado oil production line at Olivado New Zealand. The flow diagram of unit operations was completed from fruit arriving from at the factory in Kerikeri, to oil stored on consumers' premises.

Once completed, each stage of the unit operations flow diagram was analysed for relative exposure to light, aqueous phase and oxygen (air). These three factors were identified as oxidation promoters. Therefore, avoiding these factors during both production and storage would maximise the oxidative quality of the oil. Points of exposure were graded on a relative scale of 0-3, 0 meaning no exposure, 3 meaning a large amount of exposure. Types of exposure for each step were then identified as being

process critical, such as exposure to water during the washing of the avocado fruit, or non-essential.

Process steps in which exposure to light, aqueous phase or air were identified as non-essential were then analysed for potential process improvement.

4 Accelerated oxidation reactor trials

4.1 *Effect of light, temperature and oxygen level on peroxide value*

Extra virgin avocado oil (EVAO) contains many minor constituents that are extracted from the fruit with the oil. Among these components are various levels of oxidation promoters such as chlorophyll and oxidation inhibitors such as tocopherols and carotenes. These components are expected to have a profound effect on the oxidative stability of the oil. The literature search revealed that exposure to light, oxygen and high temperatures had an effected the oxidation rates of oils (Rawls and Van Santen, 1970; Korycka-Dahl and Richardson, 1978; Hamilton, 1994). The effects of these three oxidation promoting factors on the oxidation of EVAO was examined and the results are shown in Figure 16.

The effects of light, temperature and oxygen level on PV change after seven hours was then determined by generating a main effects plot using MINITAB software (Figure 17). This effects plot shows the average effect each of the three factors has on the PV of EVAO.

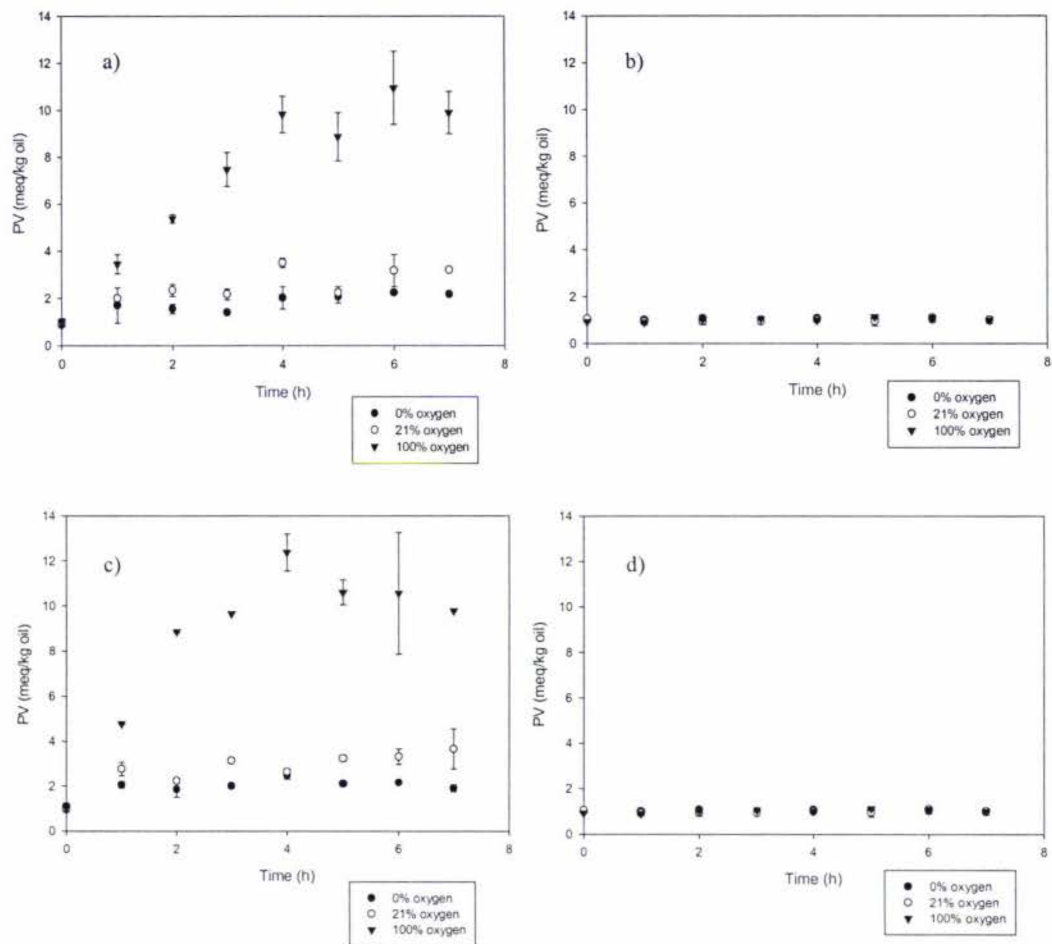

Figure 16. Effect of light, temperature and oxygen levels on the PV of EVAO: a) EVAO oxidised at 60°C, 4500 lux, varying oxygen level; b) EVAO oxidised at 60°C, 0 lux, varying oxygen level; c) EVAO oxidised at 25°C, 4500 lux, varying oxygen level d) EVAO oxidised at 25°C, 0 lux, varying oxygen level

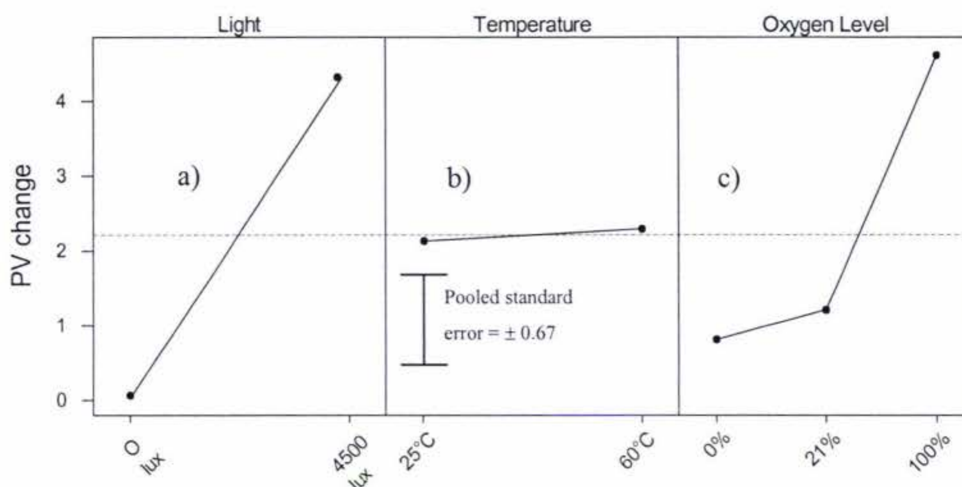

Figure 17. Main effects plot for light, temperature and oxygen level on PV change: a) Effect of 0 lux vs. 4500 lux fluorescent light on PV; b) Effect of 25°C vs. 60°C on PV change; c) Effect of 0% vs. 21% vs. 100% oxygen level on PV change

The increase in the PV of EVAO was strongly influenced by light levels. This can be seen by comparing Figure 16a and b and also in the effects plot in Figure 17a. The average effect of 4500 lux fluorescent light intensity compared to 0 lux for all treatments over a seven hour period was an increase in PV of 4.5 ± 1.4 meq/kg oil. This increase was large considering EVAO is at the end of its saleable shelf life when it reaches a PV of 10 meq/kg oil (Eyres, 2001). The Codex Alimentarius guidelines for the PV of extra virgin olive oil is that PV should be less than 20 meq/kg oil (Codex, 2000).

Oxidation of EVAO exposed to light is due to the chlorophyll sensitised (type 2) photooxidation of the oil (Rawls and Van Santen, 1970, Werman and Neeman 1986b). This reaction involves excitement of chlorophyll by a light source to a high-energy triplet state, followed by transfer of this energy to oxygen, which returns the chlorophyll to its ground energy state. The oxygen becomes excited to a singlet state and reacts directly with unsaturated bonds in the lipid molecule causing hydroperoxide formation (Korycka-Dahl and Richardson, 1978). These hydroperoxides are measured by the PV test and can break down to catalyse autoxidation or form secondary oxidation products.

When isolated as a singular effect, a temperature of 60°C was found to have very little effect on the increase of PV of EVAO compared to 25°C. The average effect of temperature for all treatments was an increase of 0.1 ± 0.1 meq/kg oil over a seven hour period, and can be seen by comparing Figure 16a and c, or examining Figure 17b. This increase in PV was less than expected considering elevated temperatures are most commonly used to conduct accelerated oxidation tests (Frankel, 1993). This is because increasing the temperature of an oil sample increases the rate of autoxidation (Frankel, 1993). There are several possible causes for the small increase in PV due to temperature. Firstly, the time period over which the change in PV of EVAO was measured was very short, only seven hours. Accelerated oxidation tests performed on RBD avocado oil took three days to register a PV increase of only 3 meq/kg oil during storage at 60°C in dark conditions (Werman and Neeman, 1986a). Secondly, EVAO contains natural antiautoxidants such as tocopherol which may protect the oil against heat induced oxidation (Eyres et al., 2001).

The PV of EVAO appears to be strongly affected by oxygen level, as shown by bubbling air through the dry oil. The PV of aerated EVAO was increased on average by 3.5 ± 1.7 meq/kg oil over a seven hour time period compared to oil stored in ambient air conditions as shown in Figure 16a and c and also in Figure 17c. Reducing the oxygen content by bubbling nitrogen through the oil had a small affect on decreasing the PV increase. This decrease in PV due to the effect of oxygen stripping (sparging) was 0.8 ± 0.4 meq/kg oil during storage over 7 hours. This may have suggested that there was only a small amount of dissolved oxygen present in the oil initially and this was displaced by the nitrogen gas. The dissolved oxygen level in the oil samples were not measured in this experiment. However, studies have shown that nitrogen gas sparging in wine products has been able to reduce dissolved oxygen levels to below 2% (Anon, 1987).

The oxygen level also appeared to have an affect on the precision of the PV test. The standard error of the PV appeared greater when oxygen was bubbled through oil sample (0.5-3.0) compared to the PV standard error of samples in oxygen at atmospheric pressure or below (0.1-1.5). These differences in standard error are shown in Figure 16a

and c, 100% oxygen. This is most likely due to the 'oxygen error', which has been reported to occur when high levels of oxygen are dissolved in an oil sample (AOCS, 1993).

An interaction plot was also generated using MINITAB software showing the interactive effects of light, temperature and oxygen level on the PV change in EVAO after 7 hours of storage and is shown in Figure 18.

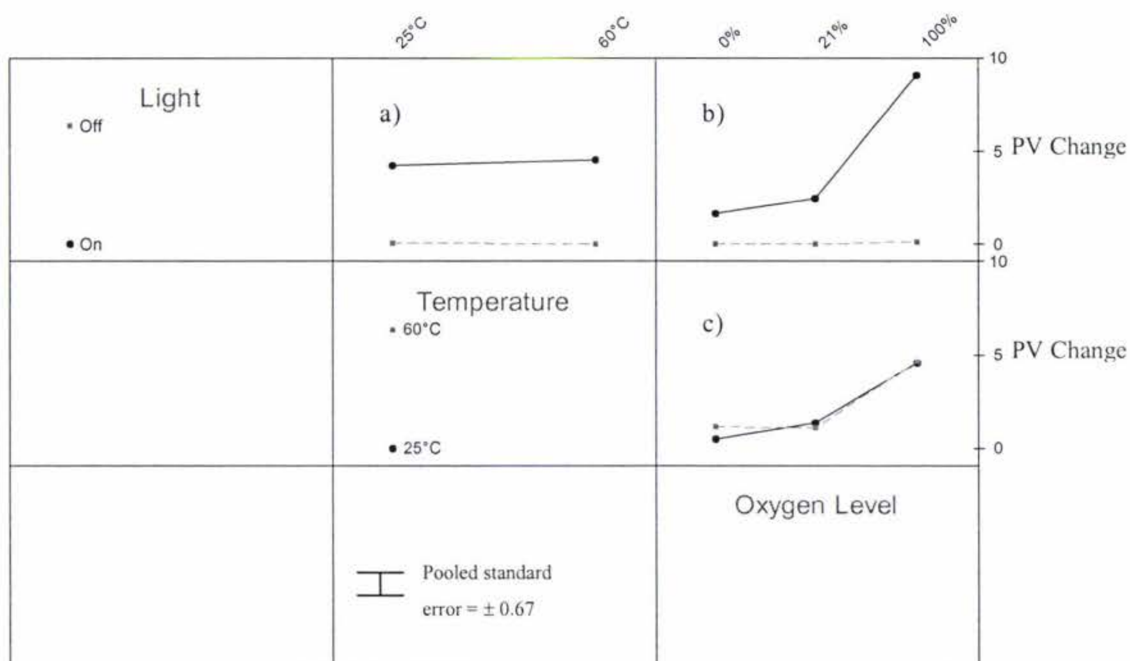

Figure 18. Interaction plot for light, temperature and oxygen level on PV change: a) Interaction between light level and temperature; b) Interaction between light and oxygen level; c) Interaction between temperature and oxygen level.

There was no apparent interaction between light and temperature on the PV increase of EVAO over a seven hour period as shown in Figure 18a. This result suggested that the oxidation reactions taking place under these two conditions were independent. Oxidation under lit conditions is due to photooxidation whereas oxidation at higher temperatures is due to autoxidation. Rawls and Van Santen (1970) also found that photooxidation and autoxidation reactions were independent, at least during the early

stages of oxidation in oil.

There was a strong interaction between light and oxygen level on the PV increase of EVAO as shown in Figure 18b. Large increases in PVs were found when high levels of oxygen were used with 4500 lux fluorescent light. This may have been because at low levels of oxygen, oxygen concentration may be a rate-determining factor in photooxidation, especially since photooxidation is very rapid. This is based on the knowledge that ground state triplet oxygen is a primary substrate in the type II photooxidation reaction found in oils containing chlorophyll (Gutierrez-Rosales et al., 1992).

There was no substantial interaction between temperature and oxygen level on the PV of EVAO as shown in Figure 18c. Unlike photooxidation, autoxidation at both 25°C and 60°C was slow – the oxygen content of the oil did not appear to be a rate-determining factoring these experiments. Thus, oxygen content had little affect on the autoxidation of EVAO at 60°C. Ragnarsson and Labuza also (1977) suggested that increasing headspace oxygen content has little effect on accelerating autoxidation at ambient temperature. However at higher temperatures such as those used in accelerated oxidation studies, the effect of oxygen presence can become considerable. The temperatures implied in this review were much higher than 25°C and 60°C used in this study. This is probably why no temperature – oxygen level interactions were detected in dark (autoxidative) oxidation in this experiment.

4.2 Effect of light, temperature and oxygen level on chlorophyll levels

EVAO contains high levels of chlorophyll at 15-60 ppm (Werman and Neeman, 1987; Eyres et al., 2001). It has been found that chlorophyll can act as a prooxidant during photooxidation when exposed to light or can act as an antiautoxidant in dark conditions (Rawls and Van Santen 1970, Endo et al., 1985a). Chlorophyll can be rapidly broken down by light and oxidation intermediates (Usuki et al., 1984; Werman and Neeman, 1986a). The total chlorophyll pigment content of EVAO was examined with respect to

respect to time based on the effects of light, elevated temperature and various oxygen levels. Results are shown in Figure 19.

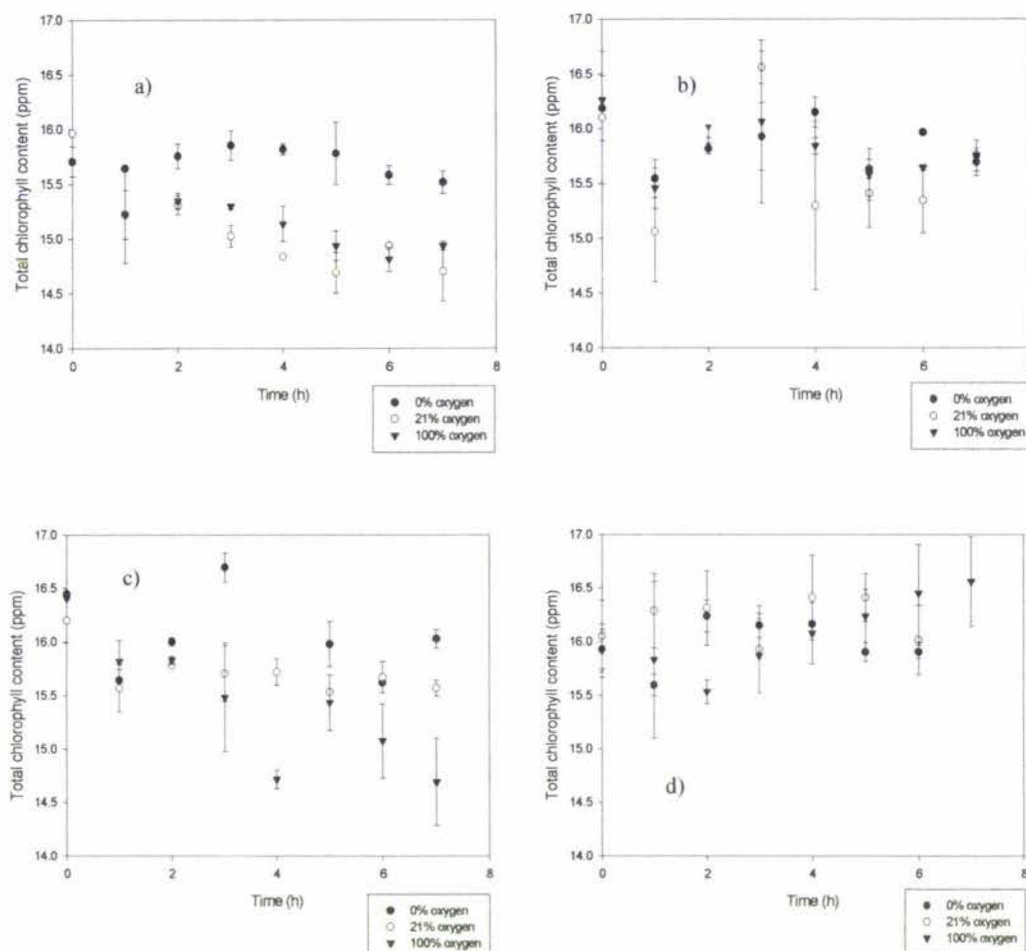

Figure 19. Effect of light, temperature and oxygen levels on chlorophyll levels in EVAO: a) EVAO oxidised at 60°C, 4500 lux, varying oxygen level; b) EVAO oxidised at 60°C, 0 lux, varying oxygen level; c) EVAO oxidised at 25°C, 4500 lux, varying oxygen level; d) EVAO oxidised at 25°C, 0 lux, varying oxygen level

The average chlorophyll content for each treatment was calculated at the beginning of the experiment and also at the end of the seven hour oxidation period based on data shown in Figure 19. A student t-test was then used to test if there was a significant difference ($p<0.05$) in chlorophyll content before and after the seven hour oxidation treatment. These results are shown in Table 5.

Table 5. Chlorophyll content change over seven hour storage for EVAO

Light intensity	Temperature	Oxygen level	Chlorophyll content (0 hours)	Chlorophyll content (7 hours)	Significant t-test difference
0 lux	25°C	0%	15.9	15.9	No ($p>0.05$)
0 lux	25°C	21%	16.1	16.0	No ($p>0.05$)
0 lux	25°C	100%	15.9	16.6	No ($p>0.05$)
0 lux	60°C	0%	16.3	15.8	No ($p>0.05$)
0 lux	60°C	21%	16.1	15.8	No ($p>0.05$)
0 lux	60°C	100%	16.3	15.8	No ($p>0.05$)
4500 lux	25°C	0%	16.4	16.0	No ($p>0.05$)
4500 lux	25°C	21%	16.2	15.6	No ($p>0.05$)
4500 lux	25°C	100%	16.4	14.7	No ($p>0.05$)
4500 lux	60°C	0%	15.7	15.5	No ($p>0.05$)
4500 lux	60°C	21%	16.0	14.7	No ($p>0.05$)
4500 lux	60°C	100%	17.3	14.9	Yes ($p<0.05$)

Only one treatment showed a significant decrease in the chlorophyll content of EVAO at $\alpha = 0.05$. The effects of light, temperature and oxygen level on chlorophyll content change after seven hours was also determined by generating a main effects plot using MINITAB software (Figure 20). This effects plot shows the average effect each of the three factors has on the chlorophyll content of EVAO.

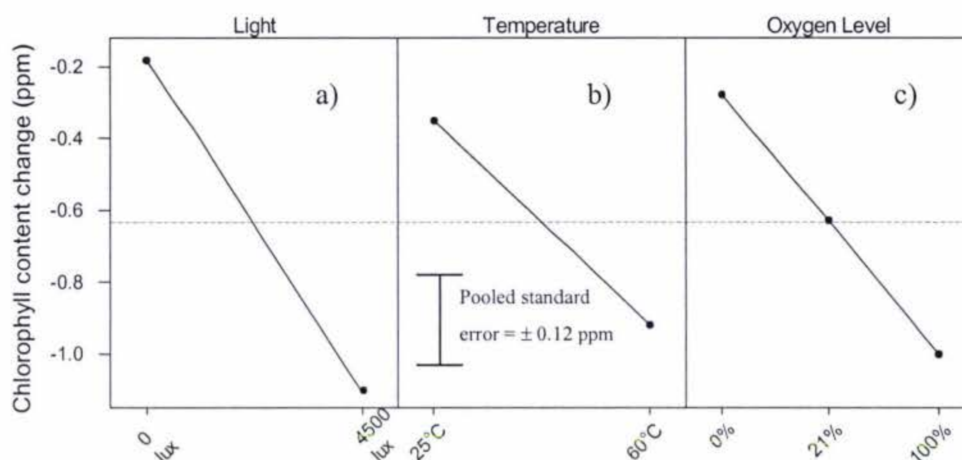

Figure 20. Main effects plot for light, temperature and oxygen on chlorophyll content change: a) Effect of 0 lux vs. 4500 lux fluorescent light on PV; b) Effect of 25°C vs. 60°C on PV change; c) Effect of 0% vs. 21% vs. 100% oxygen level on PV change

Light had a relatively strong affect on the reduction of chlorophyll in EVAO. Over a seven hour time period, the average effect of 4500lux fluorescent light on chlorophyll concentration for all treatments was a decrease of 0.9 ± 0.3 ppm chlorophyll as shown in Figure 20a. Several researchers have reported a reduction of chlorophyll in oils due to light exposure (Usuki et al., 1984; Kiristakis and Dugan, 1985; Werman and Neeman 1986a; Fakourelis et al., 1987). Chlorophyll can be broken down due to the direct effect of visible light (Usuki et al., 1984). Both singlet oxygen and hydroperoxides are intermediates of type 2 photooxidation. This type of oxidation occurs in oils containing chlorophyll that are exposed to light. The reduction in chlorophyll in these EVAO samples may also have been partially due to break down by peroxides or singlet oxygen (Endo et al., 1985a).

Increasing the temperature from 25°C to 60°C decreased chlorophyll content in EVAO stored for seven hours by an average (for all treatments) of 0.6 ± 0.2 ppm as shown in Figure 20b. Endo et al. (1985a) found that both chlorophyll and pheophytin (both of which constitute total chlorophyll pigments) were broken down above 50°C.

Singlet oxygen or peroxides formed in photooxidation or autoxidation may have also caused the breakdown of chlorophyll pigments at high temperatures (Endo et al., 1984a; Usuki et al., 1984; Werman and Neeman, 1986a).

Chlorophyll degradation in EVAO was also affected by oxygen level where, over a seven hour storage period, it was decreased on average (for all treatments) by 0.3 ± 0.25 ppm between 0 and 21% oxygen or 0.6 ± 0.2 ppm between 0 and 100% oxygen. The overlap of the standard errors for chlorophyll change as shown in Figure 20c suggest that there is no significant difference in chlorophyll degradation between 21 and 100% oxygen. The effect of oxygen level on chlorophyll content suggests that the chlorophyll breakdown pathway is partially reliant on the presence of oxygen, since adding oxygen by compressed air increased the rate of chlorophyll breakdown and removing oxygen with nitrogen appeared to decrease the rate. It was also found that oxygen level had a large effect on the PV of EVAO. The decrease in chlorophyll content may be a downstream effect of the PV increase: peroxides and singlet oxygen formed during oxidation at high oxygen levels may have been responsible for the decrease in chlorophyll content (Endo et al., 1985a).

An interaction plot was generated using MINITAB software that showed the interactive effects of light, temperature and oxygen level on the chlorophyll content change in EVAO after seven hours of storage. This plot is shown in Figure 21.

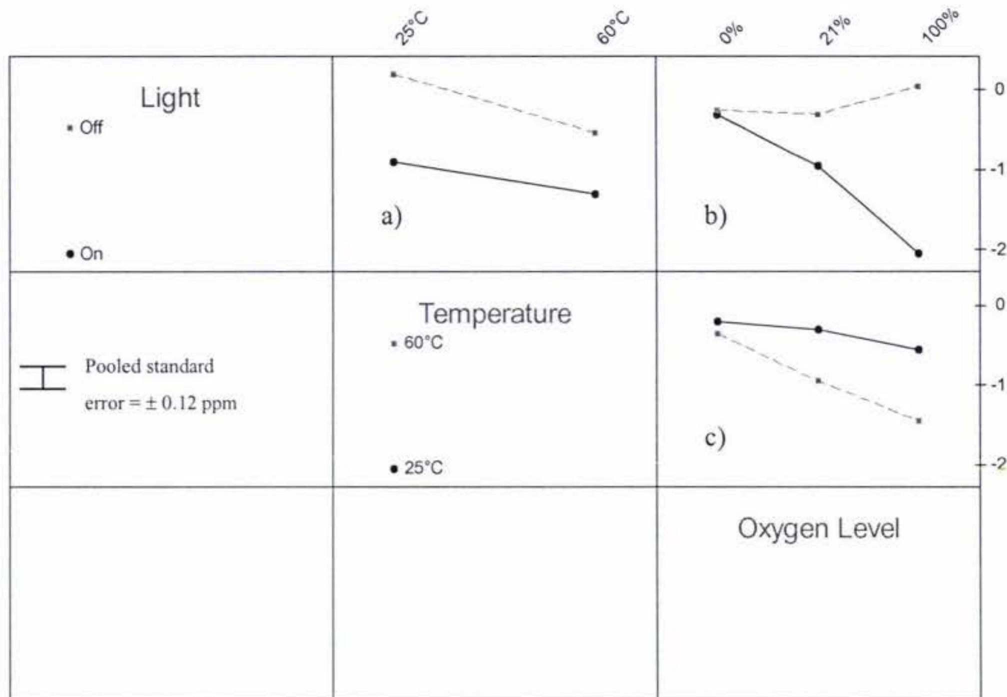

Figure 21. Interaction plot for light, temperature and oxygen level on chlorophyll content change: a) Interaction between light level and temperature; b) Interaction between light and oxygen level; c) Interaction between temperature and oxygen level.

There was no apparent interaction between light and temperature on chlorophyll levels in EVAO over a seven hour storage period as shown in Figure 21a. This suggests the effect of temperature was independent to the effect of light as was the case with PV increase (Figure 18a)

There was a strong interaction between fluorescent light and oxygen level on the effect of chlorophyll decrease in EVAO, with a large reduction of chlorophyll under fluorescent light for oxygen levels at atmospheric and above (Figure 21b). A similar interactive effect was also found for the increase of photooxidation in EVAO samples as shown by the PV increase in Figure 18b. Since chlorophyll degradation is known to be caused by reaction with photooxidation intermediates singlet oxygen and peroxide, the large decrease in chlorophyll content at 4500 lux light intensity and 100% oxygen content is most likely due to the large increase in photooxidation (Usuki et al., 1984;

Werman and Neeman, 1986a).

There appeared to be a moderate interaction between temperature and oxygen level on the effect of chlorophyll decrease in EVAO after seven hours (Figure 21c). Chlorophyll degradation was enhanced at 60°C at 21% and 100% oxygen levels. Chlorophyll pigments are broken down at temperatures above 50°C (Usuki et al., 1984). Since high oxygen content appeared to increase this breakdown rate, oxygen may be implicated in this reaction.

4.3 Discussion

Oxidation in EVAO as measured by peroxide value was strongly influenced by fluorescent light at an intensity of 4500 lux. This result was the same as the findings of Werman and Neeman (1986b) who also found a rapid increase in the PV of EVAO stored under fluorescent light. This phenomenon is common in oils containing chlorophyll that are exposed to light and has been identified as type II singlet oxygen photooxidation (Rawls and Van Santen, 1970; Korycka-Dahl and Richardson, 1978). Fluorescent light also caused a decrease in the chlorophyll content of EVAO. Werman and Neeman (1986b) also found that fluorescent light caused a rapid decrease in the chlorophyll content of EVAO. The decrease of chlorophyll content in oils that are exposed to light is due to breakdown of the chlorophyll. Chlorophyll breakdown is caused by the direct effect of the light itself or by reaction with intermediate products of photooxidation, specifically peroxides and singlet oxygen (Usuki et al, 1984; Endo et al., 1985a; Werman and Neeman, 1986b).

The effect of an elevated temperature of 60°C compared to 25°C on the oxidation rate of EVAO was small. This is in part due to the relatively stable fatty acid profile of avocado oil which consists of predominantly monounsaturated fatty acids (Eyres et al., 2001). Also chlorophyll and other natural components in EVAO may have acted as antiautoxidants in dark conditions (Rawls and Van Santen, 1970; Endo et al., 1985a; Werman and Neeman, 1986b). The elevated temperature of 60°C also had little effect on chlorophyll content decrease over seven hours. Endo et al. (1985a) found that

chlorophyll pigments were broken down at temperatures greater than 50°C, however this rate must have been too slow to measure over seven hours at 60°C.

Oxygen level had a strong effect on PV increase in EVAO; removal of oxygen by bubbling nitrogen gas through the sample caused a reduction in PV increase whereas aeration of the sample increased the rate of PV formation. Since oxygen is involved in both photooxidation and autoxidation reactions (Hamilton, 1994), the oxygen content of EVAO may be a rate-determining factor for both types of oxidation. Oxygen level especially increased the rate of photooxidation. Korycka-Dahl and Richardson (1978) showed that formation of singlet oxygen during photooxidation requires the presence of triplet oxygen as a substrate. Therefore, the presence of oxygen in an oil sample would strongly influence the rate of singlet oxygen formation during photooxidation. Chlorophyll loss occurred in conjunction with PV increase at high oxygen levels. This was probably due to degradation by photooxidation intermediate products (Usuki et al., 1984).

The combined effect of high temperature and high oxygen levels caused a notable loss in chlorophyll content but not in PV increase over seven hours. This may have suggested that chlorophyll was preferentially broken down instead of peroxide formation. This agrees with research performed by Endo et al., (1985a) who found that chlorophyll acted as a hydrogen donating antiautoxidant which inhibited the propagation of lipid free radicals and subsequently reduced peroxide formation in oil stored in dark conditions. Another explanation for this occurrence is that chlorophyll pigments have been found to break down at the test temperature of 60°C, whereas formation of peroxides at 60°C by autoxidation is too slow to measure over a seven hour time frame.

5 Maximising oxidative stability of EVAO by reducing exposure to prooxidants

5.1 EVAO prooxidant factors

From results in section 4, it was found that fluorescent light had a very strong effect on the quality of EVAO, as measured by PV increase and chlorophyll content decrease, even over a time period of seven hours. It was concluded that minimisation of light exposure at all stages in the production and storage of EVAO was important to maintaining oil quality. Oxygen level also had a very strong affect on both PV increase and chlorophyll degradation. Therefore, it is also important to minimise oxygen exposure to the oil. Temperature appeared to have less effect on the oxidation of the oil. Thus, oil exposed to temperatures of 60°C and below would undergo little degradation during the processing time frame.

Reed et al. (2001) also reported that exposure of olive oil to fruit sediments from production could also accelerate the ultimate rate of oxidation. This is because the aqueous fruit sediment may contains enzymes which catalyse the degradation of the oil; specifically lipase which catalyses lipid hydrolysis and lipoxygenase which catalyses lipid oxidation (Eskin and Grossman, 1977; Hamilton 1994; Georgalaki et al., 1998). (Marcus et al., 1988). Therefore, it is also important to consider oil contact with the aqueous phase, which includes water and fruit sediment, during production and storage. During the production of avocado oil, fruit sediment is the pulp solids that remain in the oil or water phases which exit the horizontal decanter.

Since the effects of light, oxygen level and exposure to aqueous matter were all considered to have a profound effect on the oxidative stability of EVAO, the production and storage of this oil were closely examined for these factors. A process flow diagram for the production and storage of EVAO at Olivado NZ in Kerikeri was recorded. The process flow diagram can be found in the appendix, pages I-VI and a pictorial flow diagram is shown in Figure 22. The flow diagram was then analysed for exposure to light, air and aqueous matter. This analysis is shown in Table 6. Exposure points were

graded on a relative scale of 1-3, 1 meaning little exposure, 3 meaning a large amount of exposure. The type of exposure was then classed as 'process critical' (i.e. could not be altered) or 'repairable'.

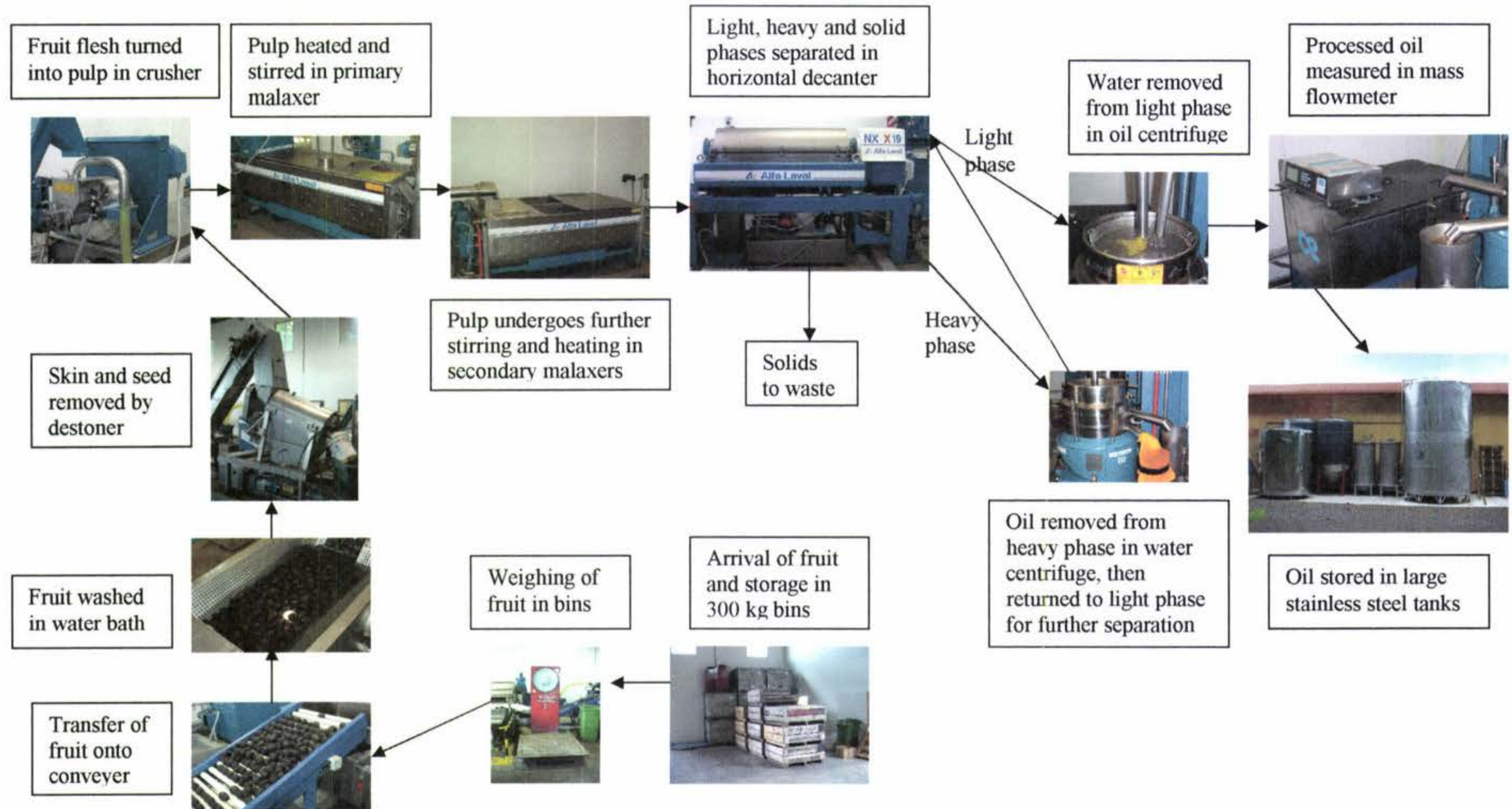

Figure 22. Pictorial process flow diagram for EVAO production at Olivado NZ

Table 6. Exposure points analysed from EVAO production and packaging at Olivado (NZ) Ltd.

Oil Production						
Step	**Process state**	**Air**	**Light**	**Water**	**Process critical/unavoidable**	**Repairable**
water bath	whole fruit			1	water	
destoner	whole fruit/flesh	2		1	air	water
flexi line from mono	flesh		1			light
disc crusher	flesh/pulp	2			air	
primary malaxer	pulp	1	1		air	light
transfer auger	pulp	2			air	
secondary malaxer	pulp	1	1		air	light
flexi line from paste pump	pulp		1			light
decanter	pulp/oil/water	3		2	water	water
collection trays	oil	1	1	1	air, water, avocado sediment	light,water
collection trays	water/oil	1	1	3	air, water, avocado sediment	light
flexi line from oil pump	oil		2			light
flexi line from water pump	water/oil		1	3		light
oil header tank	oil			2		avocado sediment
water header tank	water/oil			3	water, avocado sediment	
oil separator	oil	3		2	air, water	
water separator	water/oil	3		3	water, air	
mass flow meter	oil	1	2	2		light, water, avocado sediment
Storage tanks 1-6	oil	1		1		air, water, avocado sediment
Oil Bottling						
Step	**Process state**	**Air**	**Light**	**Water**	**Process critical/unavoidable**	**Repairable**
flexi line from storage	oil		2			light
1st inspection chamber	oil		3			light
flexi line to bottling heads	oil		1			light
overflow tank	oil		3			light
sealing of caps	oil	1				air
retail display	bottled oil		1			light
consumer storage (opened)	bottled oil	3	2			air, light

5.2 Recommendations to reduce exposure of EVAO to prooxidants during production and packaging

Analysis of EVAO production and packaging at Olivado NZ showed that there were several points during production and packaging that exposure to light, water or air was not process critical nor unavoidable, i.e. repairable.

During destoning and skin removal, water was sprayed onto the destoning machine by a spray system, which was manually regulated by the operator. This spray was performed at regular intervals to ensure the machine did not become blocked by fruit pieces. Since regulation of the cleaning water was performed manually, there was a lot of variation in the amount of water used. This method has since been automated, where water is now sprayed for 10 seconds every 2 minutes, minimising the volume of water used.

Fruit flesh is pumped from underneath the destoner to the crusher via 6m of 55mm diameter clear plastic flexible line. Although the residence time of the fruit flesh in clear line is only 10 seconds, exposure to light is significant and may initiate photooxidation in the oil contained within the fruit flesh (Werman and Neeman, 1986b). Changing this line to an opaque or black pipe would eliminate exposure to light. The disc crusher was originally designed to grind olive cells and stones to facilitate the release of oil during olive oil production (Kiritsakis, 1998). In avocado oil production, it performs a similar function except that most skin and seed particles are removed prior to crushing in the destoner. Due to the high speed and shear at which the crusher operates, a lot of air is forced into the fruit pulp and oil droplets in the avocado matrix may be emulsified (Walstra, 1996).

Low speed milling machines are still used instead of modern disc crushers in olive oil processing systems (Kiritsakis, 1998). A low speed milling machine provides the crushing effect required to break the fruit cells but with much less shear force, suggesting less aeration and emulsification compared to the high speed disc crusher. A low speed milling machine may also be considered instead of the high speed crusher for EVAO production which could potentially minimise aeration and increase the

oxidative quality of the oil produced. However, olive fruit that are processed in the crusher still contain fruit stones and skin which aid abrasion whereas avocados that are processed in the crusher have already had skin and seed removed. Therefore, further research would be required before employing a low speed milling machine instead of a high speed disc crusher in EVAO production.

During the filling and emptying of the primary malaxer with avocado pulp, the stainless steel cover is often removed for inspection and the pulp is exposed to artificial lighting within the factory. This lid can be kept closed and the hinged inspection ports can be used to inspect malaxer level, thereby minimising light exposure and potential photooxidation in the oil (Werman and Neeman 1986b).

Heated avocado pulp is transferred from the primary malaxer to one of two secondary malaxers by an auger system. This system forces a lot of air through the pulp. This pumping system also does not completely empty the primary malaxer following rinsing. The operating parameters of the transfer auger cannot easily be modified. If this pumping system was changed to a positive displacement pump, air entrapment may be avoided and much better hygiene could be achieved.

The stainless steel covers on the secondary malaxers are also regularly removed during filling and emptying, exposing the avocado pulp to the factory lights. These lids should be closed where possible and opened only for cleaning purposes. Hinged lids could be constructed to cover the inspection ports when not in use. This would minimise photooxidation due to light exposure (Werman and Neeman, 1986b).

The line connecting the paste pump at the end of the secondary malaxers to the decanter is also a 55mm diameter clear flexible line that is 6 metres long. Since inspection is not required at this stage, this pipe could be replaced with a similar black or opaque pipe that does not allow light through.

The majority of water used in oil production is added to the avocado pulp just prior to being separated in the three-phase decanter, in order to aid separation. The water flow

rate is regulated by the operator. The volumetric flow rate of the water is chosen based on the viscosity of the aqueous heavy phase that exits the decanter, which varies due to the quality of the fruit. Sifi et al. (2001) suggested that minimal water during the production of vegetable oil would maximise the quality of the product. However, the water is in contact with the pulp for only a short time period since it is separated almost immediately after being added. Therefore, the effect of water at this stage on oil quality is assumed to be minimal.

The horizontal decanter separates the avocado pulp into three phases: the light phase which consists mostly of oil, the heavy phase which consists mostly of water and the solid phase which consists mostly of exhausted avocado pulp, skin and stone particles. The light phase exits the decanter from a short section of stainless steel piping into a collection tray beneath the decanter. The light phase collection tray is exposed to water used to flush the decanter as well as some of the aqueous fruit sediment that is not completely separated in the decanter. Cleaning and drying this tray daily will minimise build up of aqueous material in the tray and exposure to the oil which may reduce enzymatic degradation and subsequent autoxidation (Reed et al., 2001). Placing a cover on the tray to reduce light exposure will also minimise photooxidation (Werman and Neeman, 1986b).

The heavy phase which consists mostly of water and fruit sediment particles, is collected in a tray beside the light phase tray underneath the decanter. This tray should also be cleaned regularly to minimise deposition of fruit solids. A water sprayer may also be added to reduce the consistency of the mixture to aid pumping. Shielding the heavy phase from light is less important than the light phase as it contains little oil.

The light phase is continuously pumped from the tray beneath the decanter to the header tank above the oil centrifuge by an oil pump. Since the pump empties the light phase tray at a rate faster than it is filled, a lot of air is incorporated into oil in the pipe it is pumped into. Changing the oil pump control to a float switch device rather than running it continuously would minimise air incorporation and reduce the rate of potential photooxidation as a result (Korycka-Dahl and Richardson, 1978).

The line that leads from the oil pump to the header tank is 12m of clear, flexible tubing. Since this line does not require inspection, it could be changed to black or opaque pipe. A small amount of aqueous matter that includes fruit sediment remains in the light phase. Deposition of these solids can occur in the header tank. Therefore, the light phase header tank should be inspected for build up of solid matter and cleaned daily to minimise exposure to the oil. Reducing exposure to fruit sediment may reduce lipid hydrolysis and oxidation rates (Reed et al., 2001).

Although the heavy phase contains very little oil, the header tank to which it is pumped above the water centrifuge should still be inspected and cleaned at least daily. This is because the heavy phase contains a lot of solid material that is deposited on the header tank walls, which may pose a hygiene problem.

The light, mostly oil phase, exits the header tank through a short stainless steel pipe and falls about 70mm through a strainer into the oil centrifuge. During this fall, it is exposed to light. A cover has been made for this step and continual use will help minimise light exposure and subsequent photooxidation in the EVAO (Werman and Neeman, 1986b). Water is added to the light phase just prior to entering the oil centrifuge. Although contact time with this water is very short, use of this 'polishing' water should be minimised since it may lead to the loss of water soluble antioxidants that protect EVAO against oxidation (Sifi et al., 2001)

'Polished' oil is then passed from the oil centrifuge to the mass flow meter through approximately 700mm of stainless steel pipe. Since the mass flow meter tank is close to the oil separator, it is exposed to a overspray water during the cleaning of the centrifuge. Lengthening this section of pipe and even placing the mass flow meter tank in a separate room would minimise exposure of oil in the tank to water during cleaning.

EVAO in the mass flow meter tank is exposed to factory lighting. A temporary cover has been made to shield the oil from the light. A more permanent hinged fixture should be made to completely block out light exposure.

Water or fruit sediment particles that pass through the oil separator can remain in contact with the oil in the mass flow meter balance tank for long periods of time. This is because the water and fruit sediment particles are essentially undisturbed in the bottom of this reservoir and can re-contaminate passing oil. Reducing the volume of this balance tank could minimise stagnation of the oil and would reduce exposure to water and sediment, thereby increasing the oxidative stability of the EVAO (Reed et al., 2001)

Another way to minimise contact of the oil with aqueous matter is to regularly drain the bottom of the reservoir, which is where the heavier aqueous matter collects. This drain line could be re-routed to rejoin the light phase in the oil separator for re-polishing. Currently, the convex bottom of the reservoir inhibits drainage of the aqueous matter by allowing the aqueous matter to pool around the sides. Redesigning the base of the reservoir to a conical shape with a drain valve at the bottom would allow efficient removal of the aqueous contaminants. Performing a purge of such a redesigned reservoir several times daily would allow efficient removal of the aqueous phase from the reservoir and minimise potential enzymatic degradation of the oil.

After nitrogen sparging, oil is pumped to one of several storage tanks. During storage, any remaining aqueous matter in the oil sediments to the bottom of the tank. Purge valves at the bottom of the cone in each tank allows this matter to be removed. This purge should be performed regularly and inspected for aqueous matter. If aqueous matter is present, it can be drained from the storage tank(s).

Oil is pumped from the storage tanks to the bottling device by a clear flexible tube. This pipe should be changed to black or opaque tubing to eliminate exposure to light and minimise photooxidation.

Before being filled into the bottling head, oil first passes into a large, clear inspection and filtration chamber that is approximately 20 litres in volume. Since the residence time of oil in this chamber is long, it should be covered with a box or some other device which prohibits exposure to light.

All of the feed and purge lines which the oil passes through in the bottling head are clear. These can all be replaced with black or opaque tubes to minimise light exposure. Only the four final 60mm sections of tube just before the bottle filling heads need to be kept clear for inspection of oil flow.

The overflow container for oil after the bottling head is clear like the inspection chamber. This can also be covered to eliminate exposure to light. Building a light cover for this container and the first inspection chamber is especially important since the residence time of avocado oil in these containers is long.

The avocado oil bottle is exposed to lights in supermarkets or retail stores. Educating retailers to store avocado oil on lower shelves away from direct light would help minimise exposure of the oil to light, since the dark green bottles are still partially permeable to light.

Labelling the bottle with ideal storage conditions ("Store in cupboard with cap on firmly") would help minimise potential exposure to light and air, thereby minimising oxidation.

6 Hot air oven accelerated shelf life testing

6.1 Background to accelerated shelf life conditions used

Avocado oil is stored in either large stainless steel vessels at the production site or in dark green glass containers at supermarkets or retailers. These storage conditions practically eliminate the exposure of avocado oil to light. The most important consideration when attempting to conduct an accelerated oxidation test is that the conditions used should be as close as possible to the conditions under which the oil is stored (Frankel, 1993; Sifi et al., 2001). Thus, light was not chosen as a factor to accelerate the oxidation of extra virgin avocado oil for shelf life tests. Oxidation via the photooxidation pathway is minimal during storage. Since autoxidation is the main route of oxidative deterioration in stored avocado oil, an increase in temperature only was chosen for shelf life acceleration. The temperature chosen for shelf life acceleration was 60°C, which is the same as used in the Schaal oven method (Kiritsakis, 1998). This was because 60°C is the maximum practical temperature at which there are minimal side reactions that may affect results in the oxidation of oil (Ragnarsson and Labuza, 1977; Frankel, 1993).

The endpoint of avocado oil shelf life was taken as the time to reach a PV of 10 meq/kg oil. This end point was much lower than using a PV of 100 meq/kg oil as suggested in the Schaal oven method as it reflected more closely the tighter quality loss parameters required for extra virgin avocado oil (Eyres, 2001). The chlorophyll content of the oil samples was also tested at the same time as PV analysis.

Colorimetric tests were also examined for use with avocado oil. Results were expressed using the Hunter Lab colour scale (Steet and Tong, 1996; Weemaes et al., 1999).

6.2 Peroxide value results and discussion

The PV of avocado oil samples were measured regularly during storage at

60°C. These results are shown in Figure 23. The samples tested were one month and nine month old EVAO and also four month old refined, bleached and deodorised (RBD) avocado oil. The initial PV and time take to reach PV 10 meq/kg oil is also shown for all three oil samples in Table 7.

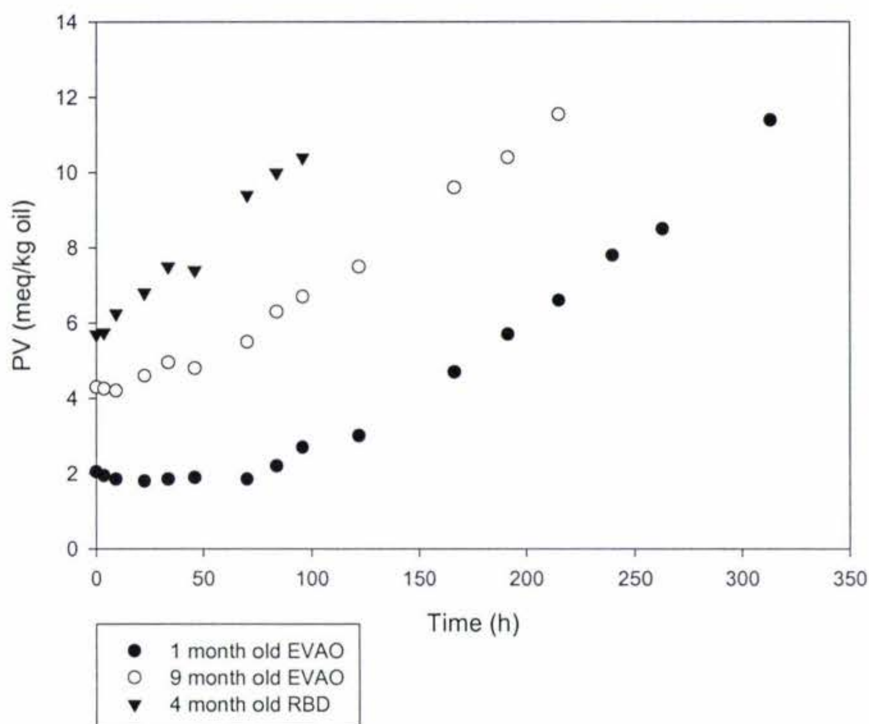

Figure 23. Effect of dark, ambient oxygen level, 60°C storage on the PV of avocado oil samples. Each point represent one PV determination. An increased frequency of PV determination was chosen in favour of replication

Table 7. Accelerated oxidation results for avocado oil stored in dark at 60°C

Oil	Time taken to reach PV 10 meq/kg oil at 60°C	Iintial PV of oil at time of testing
1 month old EVAO	297 hours	2.1 meq/kg oil
9 month old EVAO	179 hours	4.3 meq/kg oil
4 month old RBD oil	83 hours	5.7 meq kg oil

Each of the three oils tested for this experiment had different starting PVs. This affected the time taken to reach a PV of 10 meq/kg oil; the higher the initial PV of the oil, the less peroxide formation was required to reach a PV of 10 meq/kg oil as shown in both Figure 23 and Table 7. Peroxides also autocatalyse autoxidation (Hamilton, 1994). Therefore, the oil samples with higher initial PVs would have greater levels of peroxides present to catalyse autoxidation.

Both EVAO samples appeared to have an induction period after which the rate of PV formation accelerated rapidly. The induction period at 60°C was approximately 50 hours in the nine month old extra virgin avocado oil sample and approximately 80 hours in the one month old EVAO sample. The presence of an induction period in these oil samples may be due to the presence of natural compounds in the extra virgin oil samples that may provide an antiautoxidant effect, such as tocopherols, β-carotene or even chlorophyll (Werman and Neeman, 1986b; Eyres et al., 2001). The reason for the difference in induction period in these two EVAO samples may be due to a greater degree of breakdown or inactivation of antiautoxidants in the older sample. Oxidation intermediates such a peroxides and singlet oxygen have been found to cause the breakdown of natural antioxidants in oil (Foote et al., 1970; Carlsson et al., 1976; Usuki et al., 1984). Thus, the higher initial peroxide content (PV) of the nine month old EVAO sample may have resulted in greater destruction of the natural antioxidants present. The higher initial peroxide content of the older EVAO sample may also have contributed to shorter induction period, since peroxides catalyse the autoxidation reaction (Hamilton, 1994).

There was no noticeable induction period in the RBD avocado oil sample as shown in Figure 23. The processes of refining, bleaching and deodorising avocado oil removes many non-lipid compounds from the oil (Nawar, 1996). It is likely that some of the compounds that provide antiautoxidant activity in EVAO were among those removed, offering the oil less protection against oxidation. The four month old RBD avocado oil had a higher initial PV suggesting it had undergone a greater degree of oxidation. Intermediate products of oxidation may have also caused destruction of natural antioxidants, such as tocopherols, that remain in RBD avocado oil following the

refining process (Foote et al., 1970; Carlsson et al., 1976; Usuki et al., 1984).

6.3 Chlorophyll content results and discussion

During hot air oven storage at 60°C, the chlorophyll content of both EVAO samples was tested regularly. A plot of this data is shown in Figure 24. The chlorophyll content of RBD avocado oil sample was not tested since it contained no detectable chlorophyll pigments and was essentially colourless (Bakels Edible Oils, New Zealand).

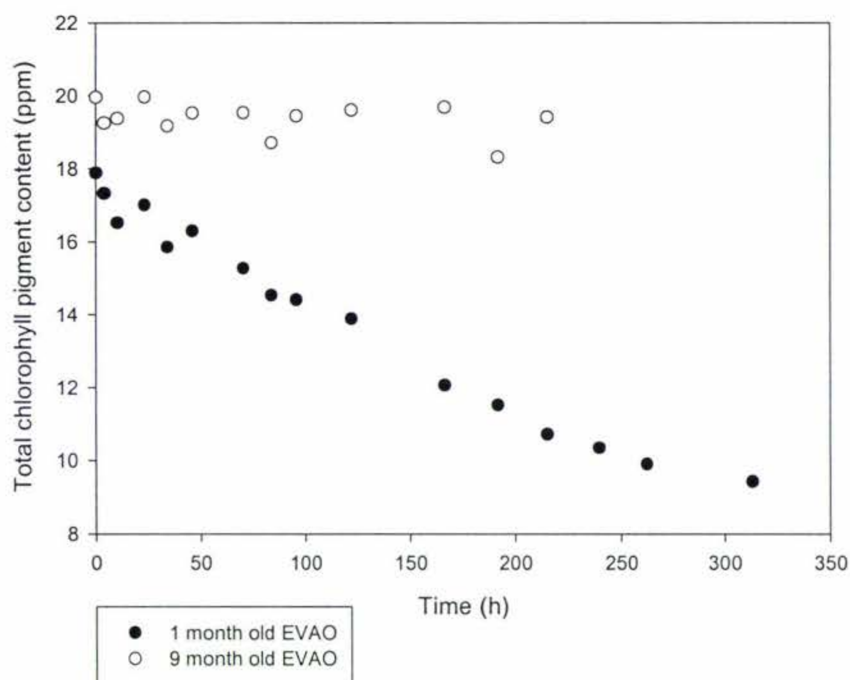

Figure 24. Effect of dark, ambient oxygen level, 60°C storage on the total chlorophyll pigment content of EVAO samples. Each point represent one chlorophyll pigment determination. An increased frequency of chlorophyll pigment determination was chosen in favour of replication.

One month old EVAO decreased in chlorophyll content from 18 ppm to 10 ppm over 300 hours of dark storage at 60°C, but 9 month old EVAO did not decrease from approximately 19ppm.

The method used to determine chlorophyll content measures total chlorophyll content; a combination of chlorophyll *a* and *b* and pheophytin *a* and *b* (AOCS, 1993). There is no conversion from chlorophyll to pheophytin in oil as conditions are unfavourable. For this reaction to occur there must be suitable enzymes present, such as those found in fresh olives, or acidic conditions (Endo et al., 1984a; Minguez-Mosquera et al., 1990). Chlorophyll *a* and *b* are less stable than pheophytin *a* and *b* (Usuki et al., 1984). Therefore, pheophytin levels in EVAO remain relatively constant with time whereas chlorophyll levels decrease. This would suggest there would be a greater proportion of pheophytin in the nine month old EVAO sample compared to the one month old EVAO sample since chlorophyll would have degraded during storage. Since the nine month old EVAO contained a greater proportion of the more stable pheophytin, the measured total chlorophyll content was relatively constant during accelerated oxidation. Usuki et al. (1984) also found that the total chlorophyll was relatively constant in soybean oil with added chlorophyll and pheophytin, owing to the stability of pheophytin.

The proportion of chlorophyll *a* and *b* compared to pheophytin *a* and *b* in one month old EVAO was much greater compared to the nine month old sample. The chlorophyll pigments measured (mostly chlorophyll *a* and *b*) were less stable at higher temperatures. This is why the total chlorophyll pigment value decreased during storage at 60°C.

EVAO has been found to range in chlorophyll pigment content, from 15-60ppm (Werman and Neeman, 1987; Eyres et al., 2001). The difference in initial chlorophyll content in the two EVAO samples could be explained by sample variation, especially since these two oil samples were produced at different times in the season.

6.4 Hunter Lab colorimetry results and discussion

Hunter Lab values for the two EVAO samples were measured regularly during storage at 60°C. The negative *a* value has been found to represent greenness in food samples containing chlorophyll (Weemaes et al., 1981; Steet and Tong, 1996). This value was plotted against storage time and is shown in Figure 25. The RBD avocado oil sample

was not tested for Hunter Lab values since it contained no chlorophyll pigments and was essentially colourless.

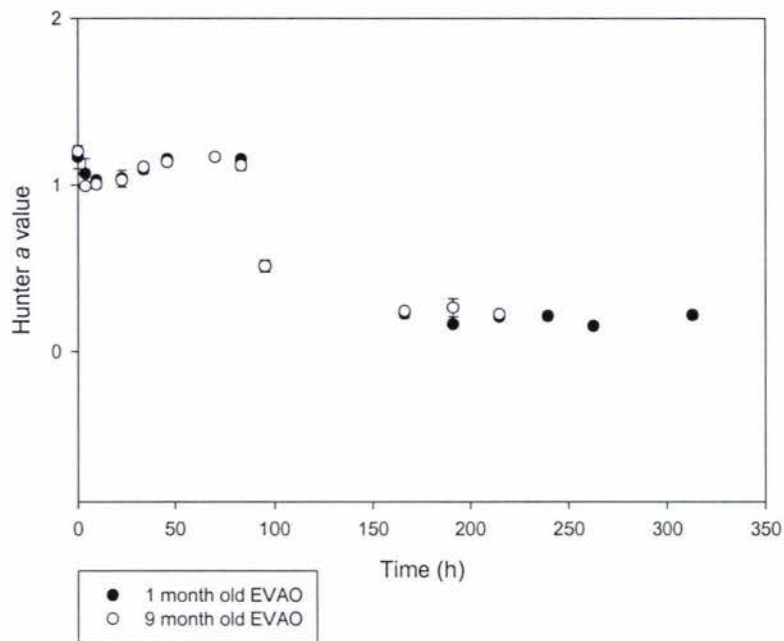

Figure 25. Effect of dark storage at 60°C on the Hunter *a* value of EVAO samples

The negative *a* value of the Hunter Lab colour scale represents green colour and has been identified as an excellent parameter for measuring the greeness of a sample (Weemaes et al., 1981; Steet and Tong, 1996). During the accelerated oxidation of extra virgin avocado oil at 60°C, the Hunter *a* value decreased from around +1.2 at 0 hours to around +0.2 at 200 hours. This decrease occurred between 80 and 170 hours of storage at 60°C. These positive values suggested that the oil samples tested were closer to the red hue/chroma and not close to the green end of the hue/chroma spectra. Visually the samples appeared bright green. The decrease in Hunter *a* value over this period did not appear to correlate with a visible decrease in green colour nor a substantial decrease in chlorophyll pigment in either 1 month or 9 month old EVAO. Also there was a visible difference as seen by the naked eye in colour between 1 month and 9 month old EVAO at all times during this experiment which was not shown by the Hunter *a* value. These results suggest that measuring only Hunter *a* values of EVAO was unsuitable for detecting the loss or even initial greeness in samples.

7 EVAO oxidation kinetics and shelf life prediction

7.1 Q_{10} Reaction kinetics

In analysing storage studies, Q_{10} values have been used to determine the affect of accelerated oxidation instead of activation energies (Ragnarsson and Labuza, 1977). The Q_{10} value is the increase in rate or decrease in shelf life of a product for a 10°C increase in temperature. For example, if an oil sample had an autoxidation Q_{10} of 2, increasing storage from 20°C to 30°C would double the rate of autoxidation and halve the shelf life.

The Q_{10} value was calculated for three avocado oil samples. It was determined by measuring the time taken for each sample to reach an end-point PV of 10 meq/kg oil during dark storage at 50 and 60°C. The avocado oil samples tested were one and ten month old EVAO and four month old RBD avocado oil. PVs were determined regularly during storage and these results and linear regression are shown for storage at 60°C in Figure 26 and storage at 50°C in Figure 27. The chlorophyll content of the one and ten month oil EVAO samples was also determined at regular intervals. Results for 50°C and 60°C storage are shown in Figure 28.

7.2 Peroxide value discussion

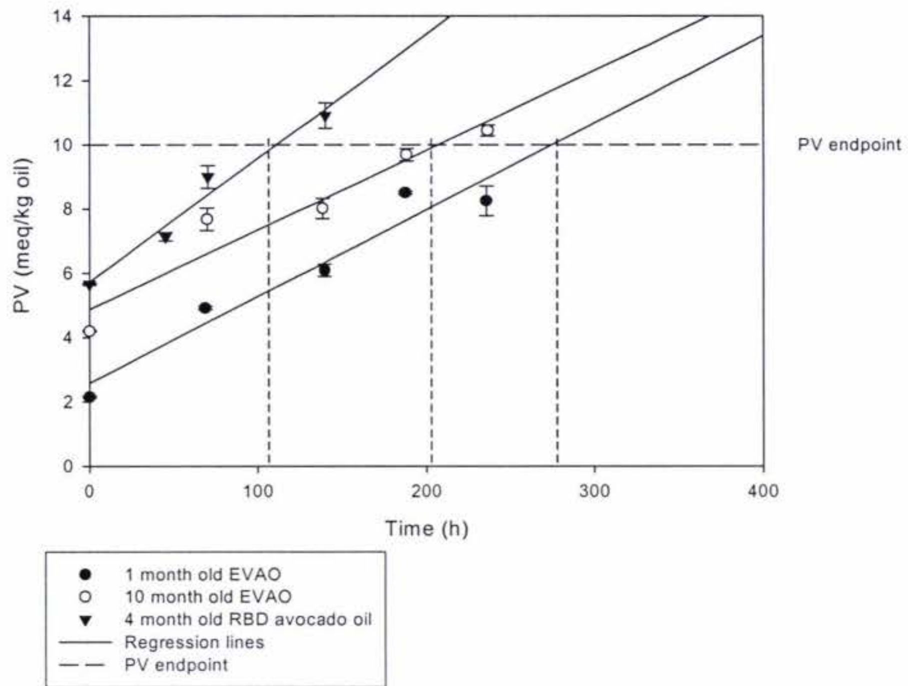

Figure 26. Effect of dark storage at 60°C on the PV of 1 and 10 month old EVAO and 4 month old RBD to determine the end of shelf life based on peroxide value

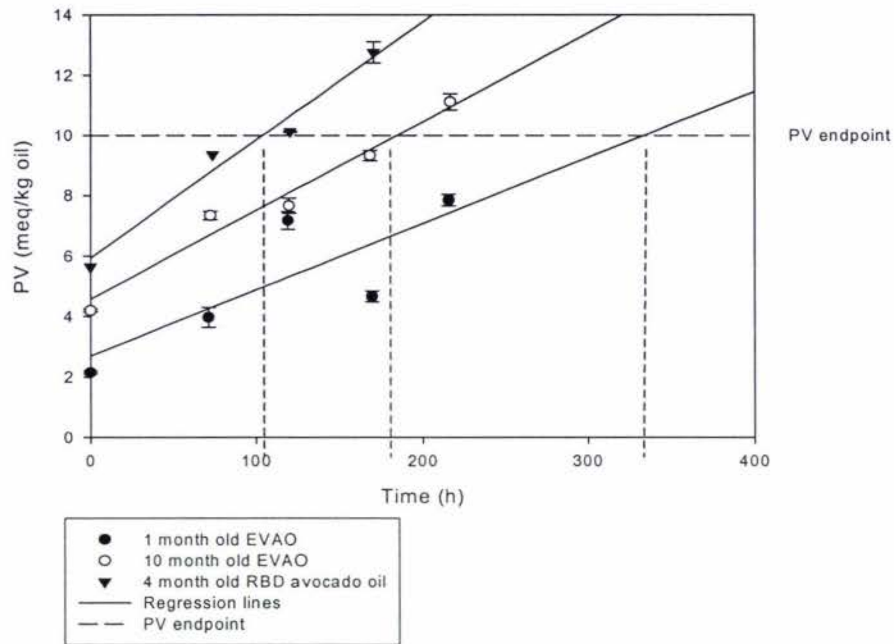

Figure 27. Effect of dark storage at 50°C on the PV of 1 and 10 month old EVAO and 4 month old RBD to determine the end of shelf life based on peroxide value

One month old EVAO stored at 60°C took the longest to increase to a PV of 10 meq/kg oil (from 2 meq/kg oil) at a projected 270 hours. This was probably due to the protection offered by natural antiautoxidants present in the oil. Ten month old EVAO took less time to reach a PV of 10 meq/kg oil from 4 meq/kg oil at around 200 hours. This probably due to a lower quantity of active antiautoxidants and a higher starting PV.

The PV of RBD oil increased significantly ($p<0.05$) from 5.5 meq/kg oil at 0 hours to 11 meq/kg oil after only 140 hours in oil stored at 60°C. This time was comparable with the 168 hours taken for PV to go from 4.4 to 16.6 in RBD avocado oil in research performed by Werman and Neeman (1986a). RBD oil had the highest PV at time 0, therefore less time at 50°C and 60°C was required to reach a PV of 10 meq/kg oil, and greater levels of peroxides would more strongly catalyse autoxidation.

The PV increase of all three oil samples was similar between the storage temperatures of 50°C and 60°C. This can be seen by comparing Figure 26 and Figure 27.

7.3 Q_{10} calculation and discussion

The Q_{10} value was calculated for all three avocado oil samples using the following equation (McCarthy, 1999):

$$Q_{10} = \frac{k_{\vartheta + 10}}{k_{\vartheta}} = \frac{t_{\vartheta}}{t_{\vartheta + 10}}$$

Where: Q_{10} = reaction rate magnitude change with 10°C temperature increase.

k_{ϑ} = reaction rate at temperature ϑ.

t_{ϑ} = shelf life at temperature ϑ.

Time taken to reach PV 10 meq/kg oil at either temperature is shown in Figure 26 and Figure 27 where the PV endpoint line intersects with the regression line for PV increase of avocado oil samples

The Q_{10} value was then used to extrapolate the shelf life of the avocado oil at 25°C (ambient temperature). Using the equation below (McCarthy, 1999):

$$\log t_s = \frac{\log Q_{10}}{10} \times \vartheta + \left(\frac{\log Q_{10}}{10} \times \vartheta_{ref} + \log t_{ref} \right)$$

Where: t_s = Shelf life at ambient temperature

θ = ambient temperature

θ_{ref} = reference temperature

t_{ref} = shelf life at θ_{ref}, the reference temperature

Calculations for the Q_{10} values and predicted shelf life at ambient temperature for each of the three oil samples are shown in the appendix, pages VI-VII. Results are shown in Table 8.

Table 8. Q_{10} values and predicted shelf life for avocado oil samples

Oil Sample	Q10 Value	Predicted shelf life at 25°C
1 month old EVAO	1.2	54 days
10 month old EVAO	0.86	2 days
4 month old RBD	1	4.5 days

These predicted times greatly under-predict the shelf life of avocado oil. For example, the 10 month old EVAO stored away from light at 25°C used in these tests had a PV of around 4 meq/kg oil whereas Q_{10} analysis predicted fresh EVAO would take just over one month to reach a PV of 10. This is probably due to the fact that a different reaction occurs at 50°C and 60°C than at ambient temperature rather than just the ambient reaction accelerated.

Rahmani and Csallany (1998) showed that carotenoid breakdown occurs at temperatures above 40°C in olive oil and loses its antioxidant activity as a result. Thus at 50 and 60°C it can be expected that the ability of carotenoids to act as antioxidants in avocado oil will be much less than at ambient temperatures.

Endo et al., (1985a) discovered that both chlorophyll and its magnesium free derivative (pheophytin) were broken down at temperatures of 50°C and above. This would create a very negative effect on the stability of the surrounding oil compared to storage at 25°C for two reasons. Both chlorophyll and pheophytin have been found to possess antiautoxidant activity in the dark (Endo et al 1985a; Endo et al., 1985a; Werman and Neeman 1986b). Destruction of these pigments due to high temperatures would cause them to loose their antiautoxidant activity and subsequently make the oil less table to autoxidation. Endo et al. (1984a) found that the oxidation products of chlorophyll were able to promote the oxidation of oils. Thus, the destruction of chlorophyll due to high temperatures may also cause the oxidation rate of EVAO to be much greater at 50/60°C than at ambient temperatures.

Ragnarsson and Labuza (1977) found that increasing headspace oxygen content has

little effect on accelerating autoxidation at ambient temperature, but at higher temperatures, such as those used in accelerated oxidation studies, the effect of oxygen presence can become considerable. During accelerated oxidation tests, avocado oil was stored in containers that were opened regularly and had a large surface area to volume ratio. Therefore they were exposed to a significant amount of oxygen whilst exposed to high temperatures and as a result, the rate of autoxidation may be expected to be greater at 50°C/60°C than at ambient temperatures.

EVAO that had been bottled directly after production and stored in the dark at ambient temperature for 15 months had a PV of 2.60 ± 0.03 meq/kg oil. The oil at the time of bottling had a PV of 0.7 meq/kg oil. This PV was very low for 14 months of storage considering EVAO is considered saleable until it reaches an end-point PV of 10 meq/kg oil (Eyres, 2001). This result is similar to that of Kiristakis and Dugan (1984) who found that extra virgin olive oil stored for 2 years in dark conditions at ambient temperature had almost no increase in peroxide value over that time.

7.4 Chlorophyll content results and discussion

Chlorophyll content in both EVAO samples was determined for oils stored at 50°C and at 60°C. These results are shown in Figure 28.

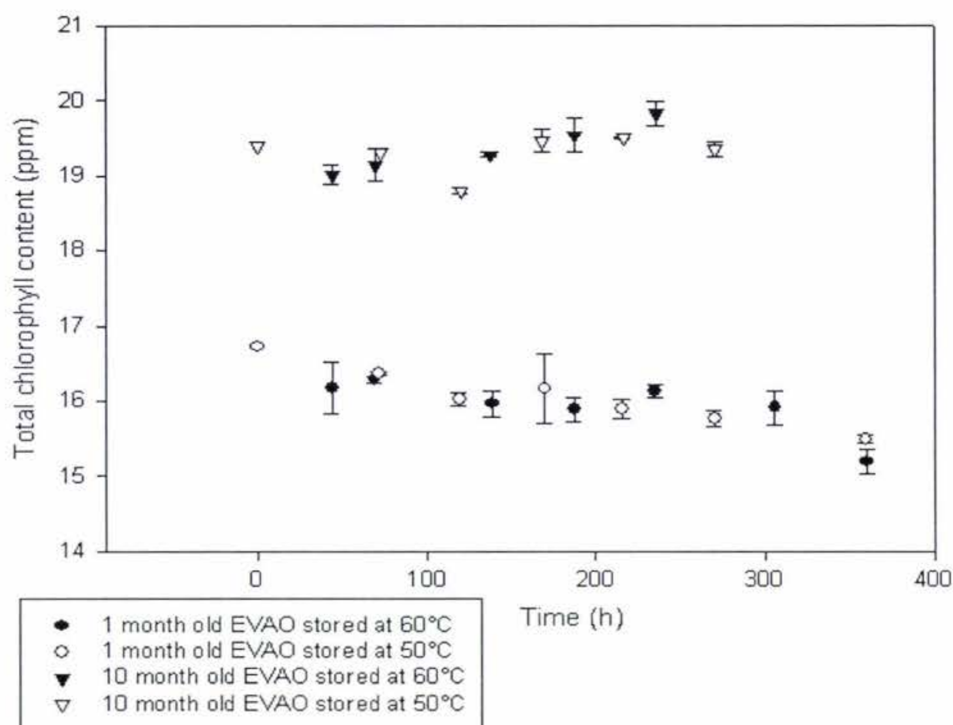

Figure 28. Effect of dark storage at 50°C and 60°C on the PV of EVAO samples

EVAO has been found to range in chlorophyll pigment content, from 15-60ppm (Werman and Neeman, 1987; Eyres et al., 2001). The difference in initial chlorophyll content in the two EVAO samples could be explained by sample variation, especially since these two oil samples were produced at different times in the season.

The chlorophyll content of 10 month EVAO stored at 50/60°C had no significant decrease during storage ($p<0.05$), remaining at around 19.5ppm during the storage period. This relationship was very similar to the one shown in the hot air oven test results (Figure 24).

The chlorophyll content of 1 month old EVAO stored at 50/60°C decreased significantly with time ($p<0.05$ for both) dropping from around 17ppm to a around 15.5ppm. This decrease in chlorophyll pigment with time was also very similar to that shown in hot air oven test results (Figure 24) since both oil samples and test conditions were the same.

8 Antioxidant evaluation in EVAO

8.1 Rancimat screening tests

Citric acid, mixed tocopherols, rosemary oleoresin and ascorbyl palmitate were all tested for their antiautoxidative efficacy in EVAO. The Rancimat oil stability index (OSI) is a rapid test for determining the relative stability of oil samples and was used as a screening method for testing antioxidants described above (Frankel, 1993). Results from Rancimat OSI analysis are shown in Table 9.

Table 9. OSI Rancimat results for EVAO containing antioxidants

Antioxidant and level in EVAO	Stability @ 100°C
Control (no added antioxidant)	21.4hr
100ppm citric acid	27.3hr
100ppm mixed tocopherols (Roche)	49.0hr
500ppm Rosemary Oleoresin (Herbor 025)	25.4hr
100ppm ascorbyl palmitate (Roche)	39.2hr

The Rancimat analysis requires a high level of oxidation for measurable results and has an unreliable endpoint (Frankel, 1993). As testing was performed at 100°C, it was expected that there may have been alternative oxidation and breakdown reactions occurring that would not normally be encountered under ambient conditions (Frankel, 1993). For these reasons, the OSI Rancimat analysis was only used as a screening test; the 60°C hot air oven was used for secondary antioxidant evaluation.

Citric acid at 100 ppm had only a very small affect on the oil stability index (OSI) of EVAO at 100°C by increasing the stability time by only 5.9 hours. However, since citric acid was being considered as a processing aid to increase the extraction efficiency of avocado oil, it was considered in the second stage of antioxidant testing (Hendriks et al., 2002).

Mixed tocopherols (100 ppm) appeared to have a very strong affect, increasing the OSI time by 27.6 hours at 100°C in EVAO. This strong antioxidative affect at 100 ppm meant that mixed tocopherols were considered in the second stage of antioxidant testing.

Rosemary oleoresin at a level of 500 ppm in EVAO had a very small affect, increasing the OSI time of the oil; only a 4.0 hour increase. This was probably because rosemary oleoresin is a large mixture of rosemary derived compounds and the components with antioxidant activity may only make up a small component of this (Hall and Cuppett, 1993). In addition, rosemary oleoresin contains compounds that are detrimental to oxidative stability such as diglycerides and monoglycerides (Hall and Cuppett, 1993). It is likely that because of these impurities and a low content of pure rosemary antioxidant, rosemary oleoresin had a much lower antioxidant affect than would be expected from pure rosemary extract.

Ascorbyl palmitate at a level of 100 ppm increased the OSI time in EVAO by 17.8 hours at 100°C. This strong affect meant that ascorbyl palmitate would be considered in the next stage of antioxidant testing.

A fourth antioxidant mixture was also chosen for secondary antioxidant evaluation in EVAO. This was a mixture of 100 ppm mixed tocopherols and 100 ppm citric acid. This mixture was chosen because these two antioxidants are said to exhibit synergy with each other – citric acid is able to 'recharge' spent tocopherols by hydrogen donation as well as chelate metal ions to enhance both antioxidants (Chang et al., 1977).

Conditions for secondary antioxidant analysis were identical to those used for Q_{10} analysis at 60°C.

8.2 Results and discussion from antioxidant tests at 60°C

Four EVAO samples containing antioxidants and a control sample were stored at 60°C

in a hot air oven in dark conditions. The PVs were determined at regular intervals. The experiment was stopped when 550 hours had elapsed. Results are shown in Figure 29.

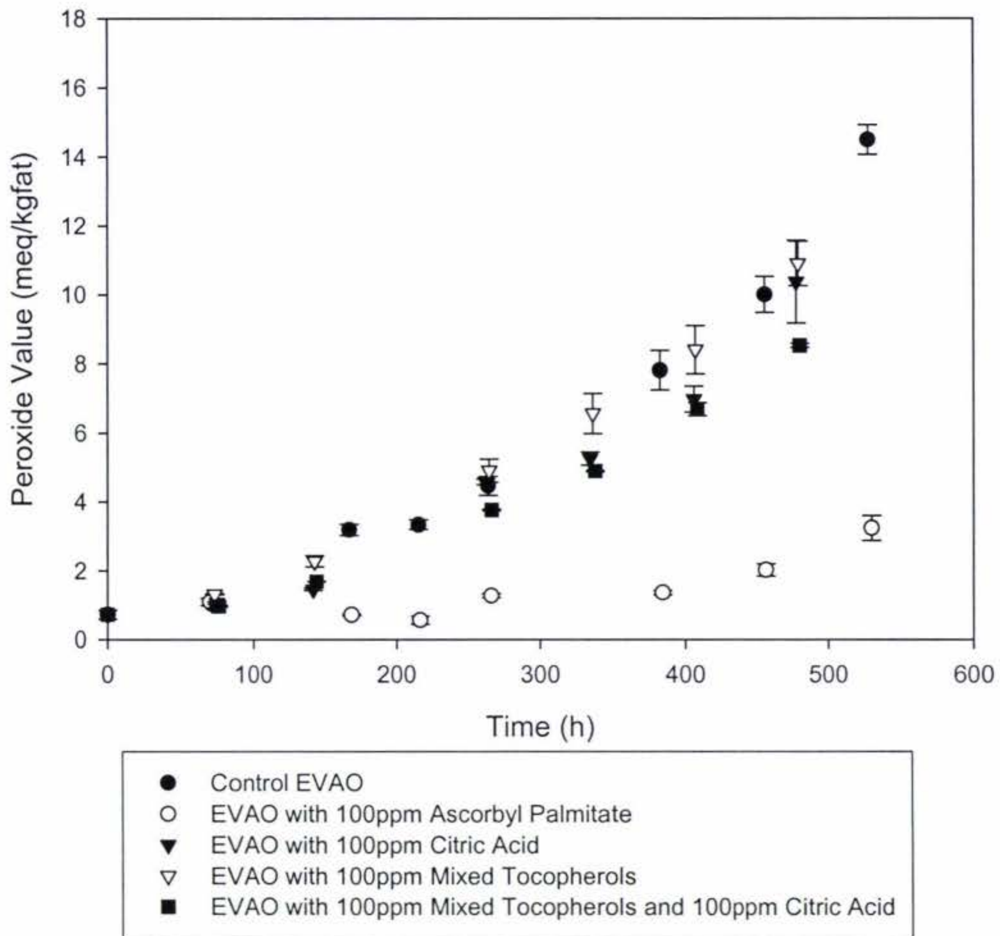

Figure 29. Effect of antioxidants on the PV of EVAO stored at 60°C in dark conditions

All oil samples tested in this experiment showed a significant increase ($p<0.05$) in PV after 550 hours of storage at 60°C. The average starting PV of all oil samples was 0.7 ± 0.1 meq/kg oil. The magnitude of the increase differed between antioxidants used.

Control EVAO increased to a PV of 12 ± 0.5 meq/kg oil after 500 hours as shown in Figure 29.

Mixed tocopherols at a level of 100 ppm appeared to have no noticeable affect on the oxidative stability of the oil when compared to the control sample, as shown in Figure 29. Tocopherols were expected to reduce the rate of PV formation in EVAO at the elevated temperature of 60°C as shown by their superior performance in OSI Rancimat analysis at 100°C. EVAO already contains 130-200 ppm total tocopherols which are extracted from the fruit during oil production (Eyres et al., 2001). Jung and Min (1990) found that the optimum concentration of α-tocopherol in stripped soybean oil undergoing autoxidation was 100 ppm, and above this level it had a prooxidant effect. A similar effect may have occurred in EVAO: adding 100 ppm mixed tocopherols to the natural tocopherols already present may have increased the total tocopherol content in the oil to a level greater than its optimum concentration. Therefore, adding 100 ppm tocopherol had no beneficial effect on the oxidative stability of EVAO and may actually have had a detrimental affect on EVAO stability.

EVAO containing 100ppm citric acid or 100ppm citric acid + 100ppm mixed tocopherols showed very similar increases in PV during storage to approximately 9 ± 1.2 meq/kg oil after 500 hours, as shown in Figure 29. The rate of PV increase for these samples was slightly less than the control sample. This antiautoxidative effect was probably due to citric acid alone, since the effect of citric acid was very similar to that of the citric acid/mixed tocopherols mixture, both of which were greater than mixed tocopherols alone. This also suggested a lack of synergy between mixed tocopherols and citric acid, in contrast to what was expected (Chang et al., 1977). The effect of citric acid on EVAO stored in dark conditions at 60°C was similar to that of OSI Rancimat testing at 100°C; a small stability improvement over the control EVAO sample containing no antioxidants.

Ascorbyl palmitate at a level of 100 ppm stood out as the most effective antiautoxidant in retarding PV formation at 60°C dark storage conditions as shown by the very slow rise in PV over time. After 500 hours of accelerated oxidation at 60°C, the peroxide value had risen to only around 3 ± 0.4 meq/kg oil (Figure 29). Most of this increase was in the last 100 hours. Unlike the mixed tocopherols, the affect of ascorbyl palmitate on the oxidative stability of EVAO was very similar to that measured in OSI Rancimat

testing at 100°C; 100ppm ascorbyl palmitate offered a considerable advantage in oxidative stability. This may suggest that ascorbyl palmitate would be effective even at ambient temperatures. Ascorbyl palmitate is highly effective in synergistic mixtures (Buettner and Jurkiewicz, 1996). Unlike citric acid, ascorbyl palmitate may have acted synergistically with natural antioxidants present in EVAO. Since ascorbyl palmitate functions as an antioxidant by scavenging oxygen, it is also suitable as an antiphotooxidant and is able to quench singlet oxygen 10 times better than α-tocopherol (Rooney et al., 1983; Lee et al., 1997).

The chlorophyll content of EVAO samples containing antioxidants was determined at the same time as PV determinations. Chlorophyll content results for these samples are shown in Figure 30.

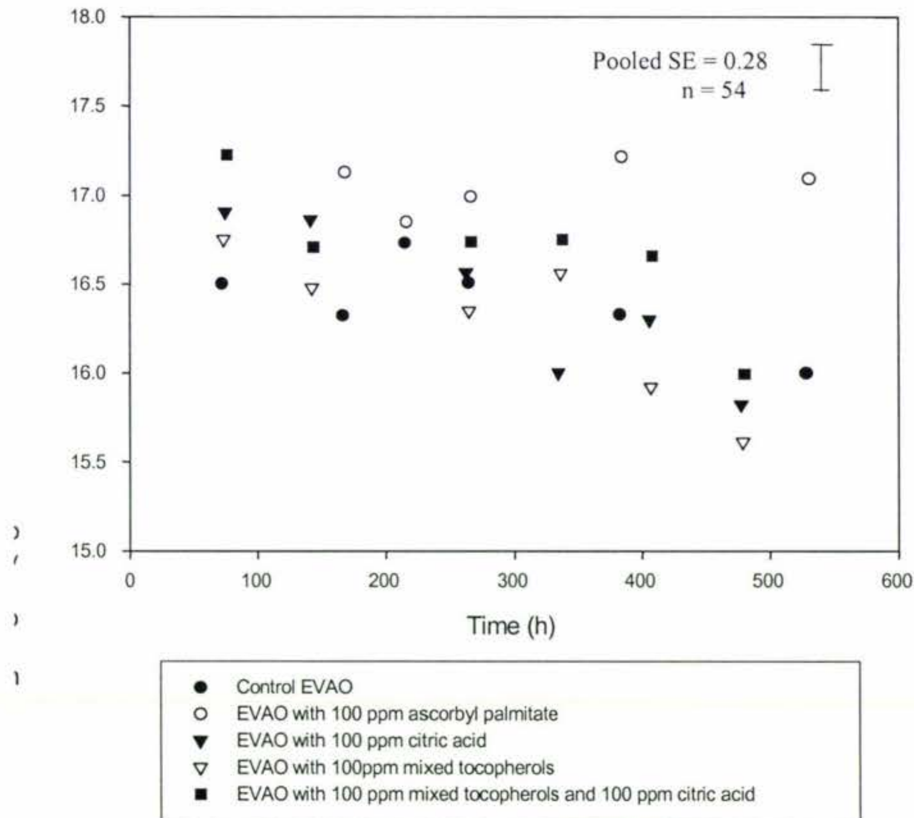

Figure 30. Effect of antioxidants on the chlorophyll content of EVAO stored at 60°C in dark conditions

Control EVAO showed no significant decrease ($p>0.05$) in chlorophyll content over 500 hours of dark storage at 60°C, as shown in Figure 30.

The EVAO sample containing 100ppm citric acid and the sample containing 100ppm mixed tocopherols both showed a significant decrease in chlorophyll content with time ($p<0.05$ for both). This may have suggested that either of these antioxidants used alone had an antagonistic affect on the destruction of chlorophyll.

The EVAO sample containing a mixture of both mixed tocopherols and citric acid did not decrease significantly with storage time at 60°C ($p=0.069$).

EVAO containing 100 ppm ascorbyl palmitate showed no significant decrease in chlorophyll content with time ($p>0.05$) which is shown in Figure 30. An addition to its ability to scavenge free radical oxygen and hydroxide species, it is also able to scavenge singlet oxygen by a quenching mechanism (Bodannes, 1979; Lee et al., 1997). Chlorophyll is rapidly broken down by singlet oxygen (Usuki et al., 1984; Baardseth and Von Elbe, 1989). Therefore, use of ascorbyl palmitate as an antioxidant may also reduce the destruction of chlorophyll, as shown in EVAO in this experiment, by quenching singlet oxygen.

9 Conclusions and Recommendations

Exposure to fluorescent light causes type 2 singlet oxygen photooxidation in extra virgin avocado oil (EVAO). Light and oxidation intermediates formed as a result of light exposure also caused a loss in total chlorophyll content in EVAO

Exposure of EVAO to 60°C had no noticeable effect on oxidation over seven hours. EVAO storage at this temperature did however cause the breakdown of chlorophyll.

High levels of oxygen caused an increase in oxidation in EVAO since ground state atmospheric oxygen is a substrate of oxidation reactions. The effect of oxygen level was especially prominent during exposure to light because photooxidation was very rapid and oxygen content was a rate-determining factor of the reaction. High levels of oxygen caused an increase in oxidation rates, resulting in more oxidation intermediates such as peroxides and singlet oxygen. These compounds also accelerated the destruction of chlorophyll in EVAO during storage.

EVAO samples stored at 60°C in dark conditions were found to have an induction period during accelerated autoxidation, where the initial rate of oxidation was very slow. This was due to the protective affect of natural antiautoxidants in the oil. Since most of the natural antioxidants are removed during the processes of refining, bleaching and deodorising, RBD avocado oil showed no induction period.

Total chlorophyll content (including chlorophyll and pheophytin pigments) measured in nine month old EVAO remained constant during accelerated oxidation at 60°C due to temperature stable pheophytin pigments. Pheophytin pigments were assumed to be present in greater proportions than chlorophyll pigments because chlorophyll pigments were broken down during nine months storage. The total chlorophyll pigment content in one month old EVAO did decrease significantly with time. This was due the greater presence of less stable chlorophyll pigments that were still present in the fresher sample.

Ascorbyl palmitate used at a level of 100 ppm in EVAO reduced the autoxidation rate at 60°C by 80 % compared to the control sample. Adding 100 ppm mixed tocopherols had no effect on increasing the oxidative stability of EVAO since natural tocopherols are already present in EVAO at the optimum level. Citric acid at 100 ppm showed a small effect on stabilising EVAO against oxidation but did not act synergistically with added tocopherol as expected.

Light exposure should be minimised during the production, packaging and storage of EVAO due to the strong affect it has on accelerating photooxidation. Aeration had a large affect on accelerating autoxidation and especially photooxidation in EVAO, it is recommended that aeration of the oil or the fruit pulp from which it is made is minimised. Exposure to water and fruit sediment particles should also be minimised since these factors ultimately accelerate the oxidation rate of EVAO.

Colorimetry analysis of EVAO using the negative *a* value of the Hunter Lab scale proved unsuccessful because of the sample measuring method used. It is recommended that this method is further developed to provide accurate and repeatable results.

It is recommended that the Q_{10} value be calculated for the oxidation of EVAO using temperatures of 30-40°C instead of 50-60°C as used in this study. Extrapolation of data from the lower temperature range to predict a shelf life at ambient temperature (25°C) would be more appropriate due to antioxidant destruction at higher temperatures.

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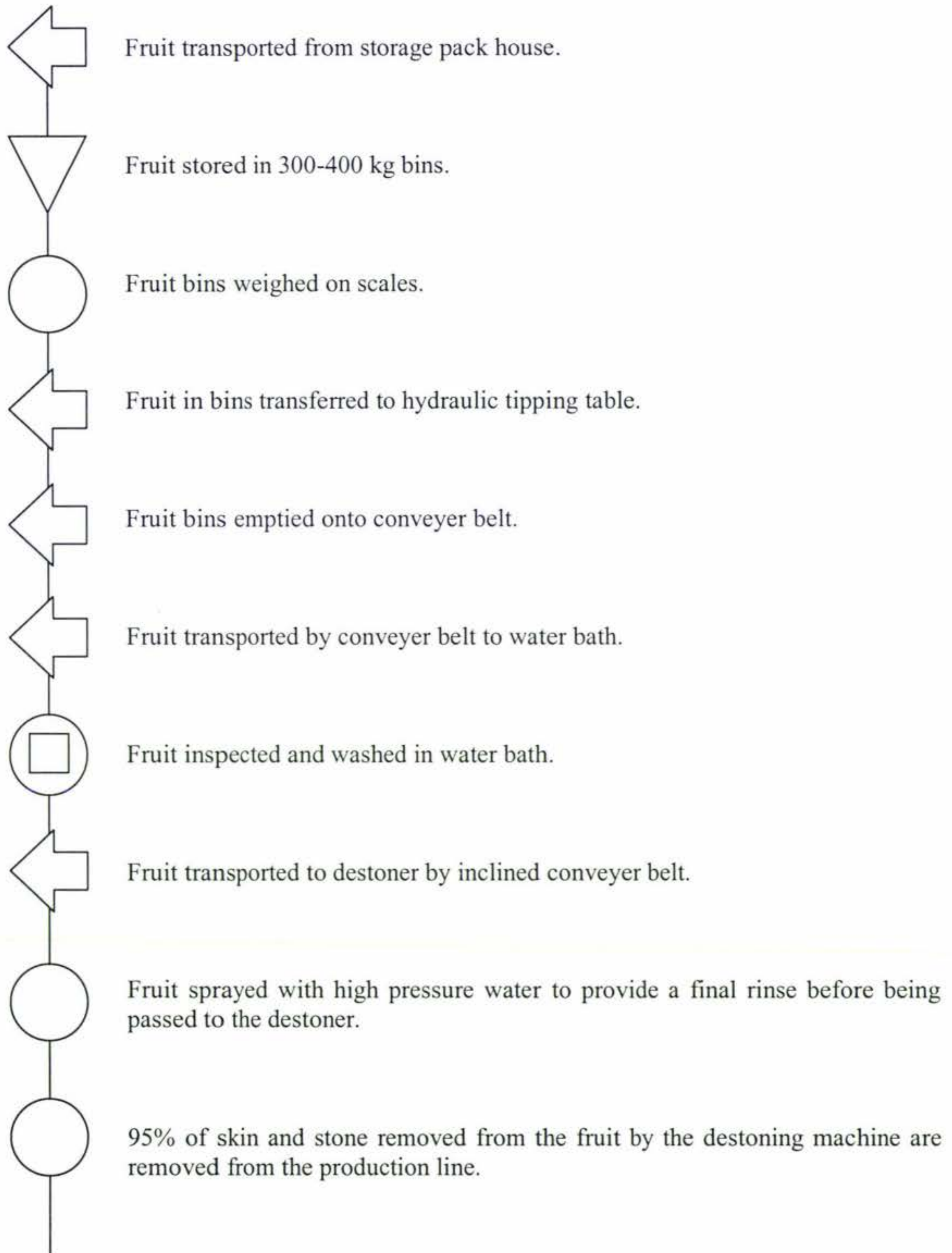
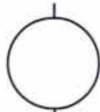
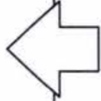
11 Appendix

11.1 Process flow diagram for the production and packaging of EVAO at Olivado NZ

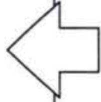




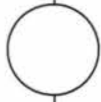
Water is sprayed on to the destoner to ensure it is kept clean, regulated automatically for 10 seconds every 10 minutes.



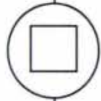
Fruit flesh and approximately 5% of skin and stone fragments that have been extruded through the destoner are then pumped through 6m of clear 55mm internal diameter flexible tube to the crusher auger.



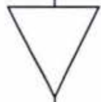
Fruit flesh pumped horizontally through a short auger to feed the disc crusher.



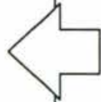
A high speed rotating disc crusher crushes the fruit flesh and skin and stone particles present. The crushed fruit flesh (now pulp) then drops into the primary malaxer.



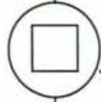
Fruit pulp is slowly stirred at 30 rpm by a ribbon blade in a heated jacket stainless steel malaxer. Pulp is inspected at this stage.

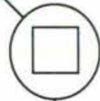


Fruit pulp is heated and stirred in the primary malaxer for about an hour.

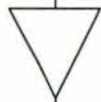


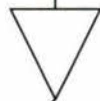
When a secondary malaxer is available, fruit pulp is then transferred to one of the two secondary malaxers by a vertical then horizontal auger.



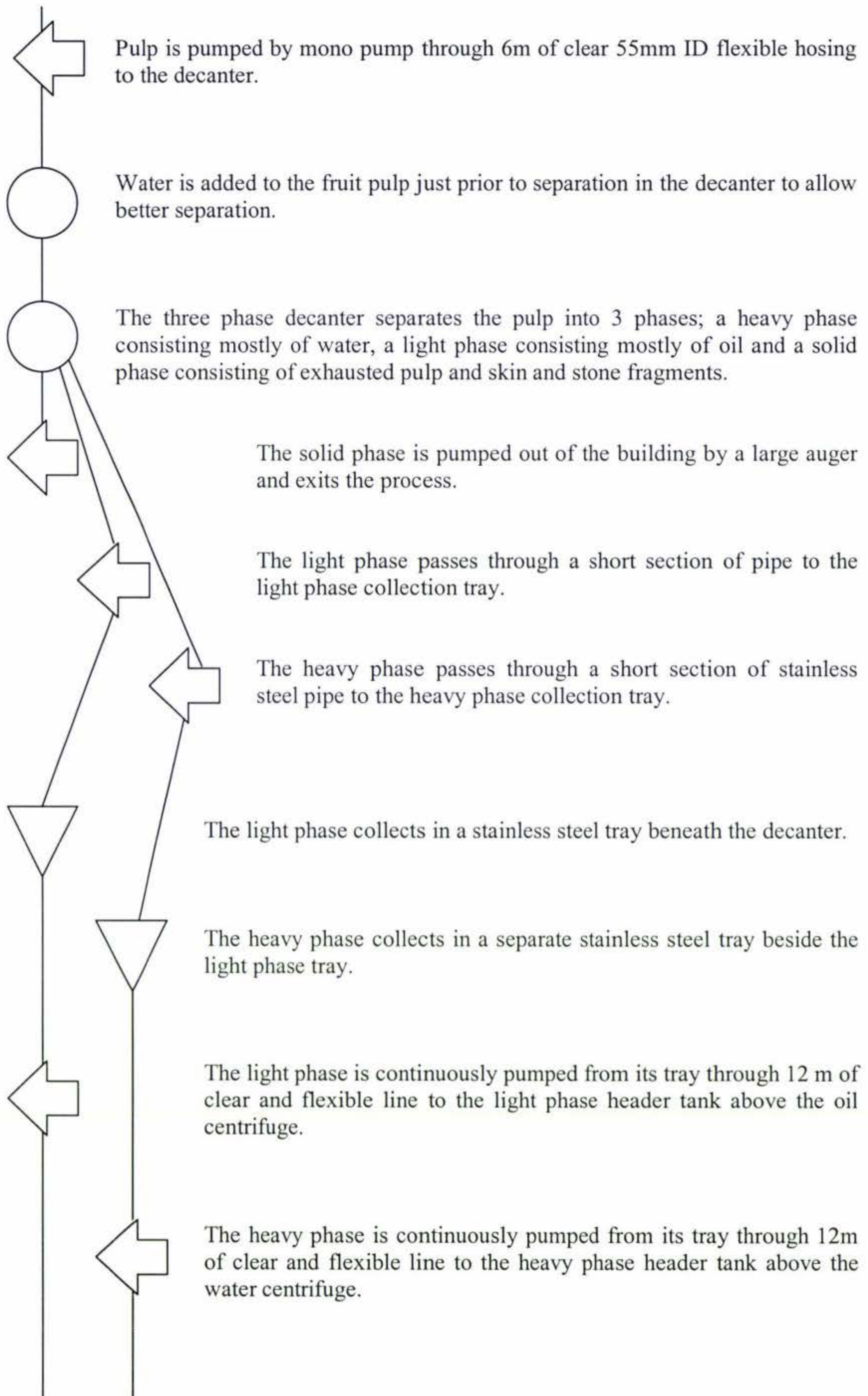


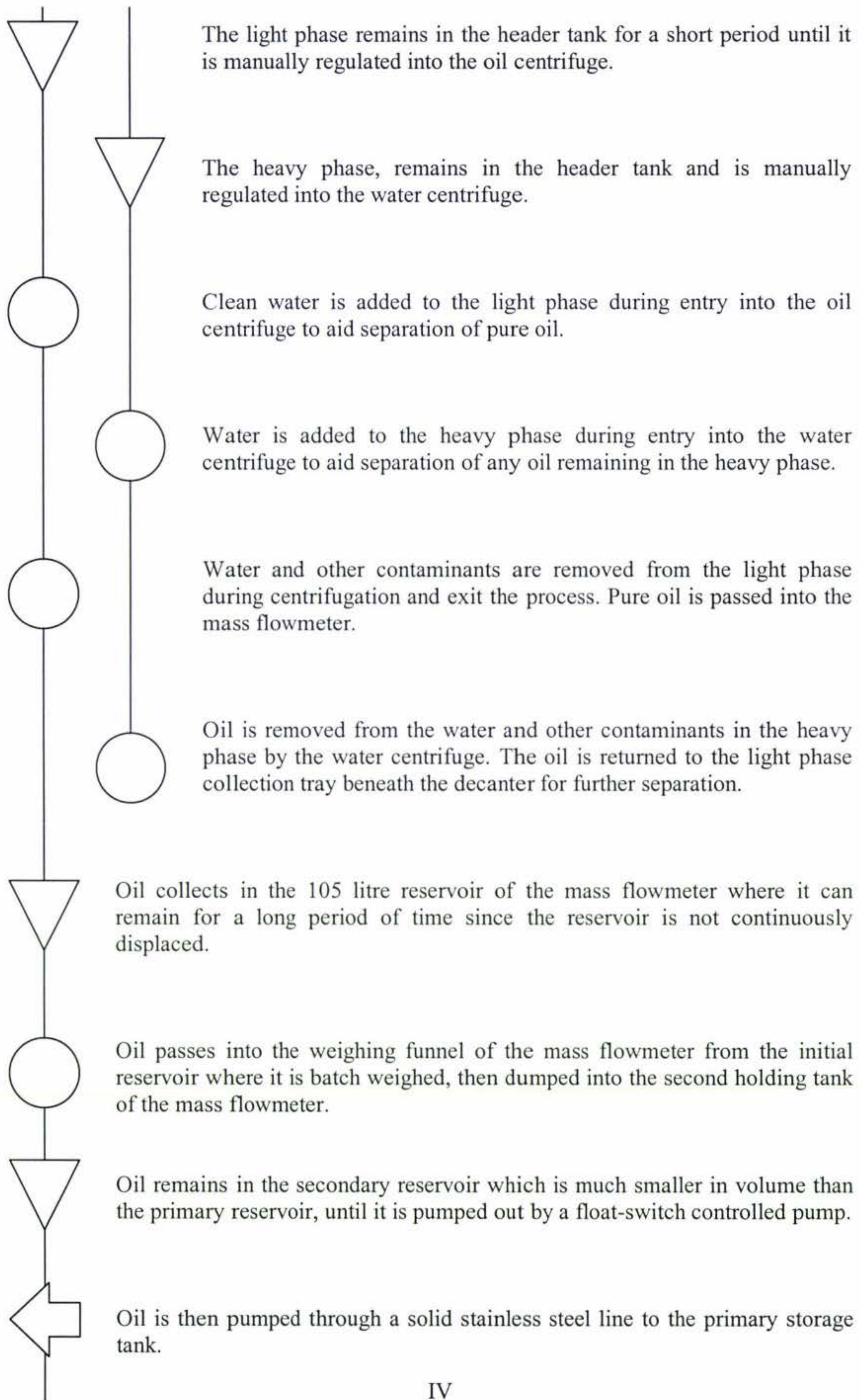
Fruit pulp is stirred and inspected for oil release in one of two secondary malaxers operating under the same conditions as the primary one.



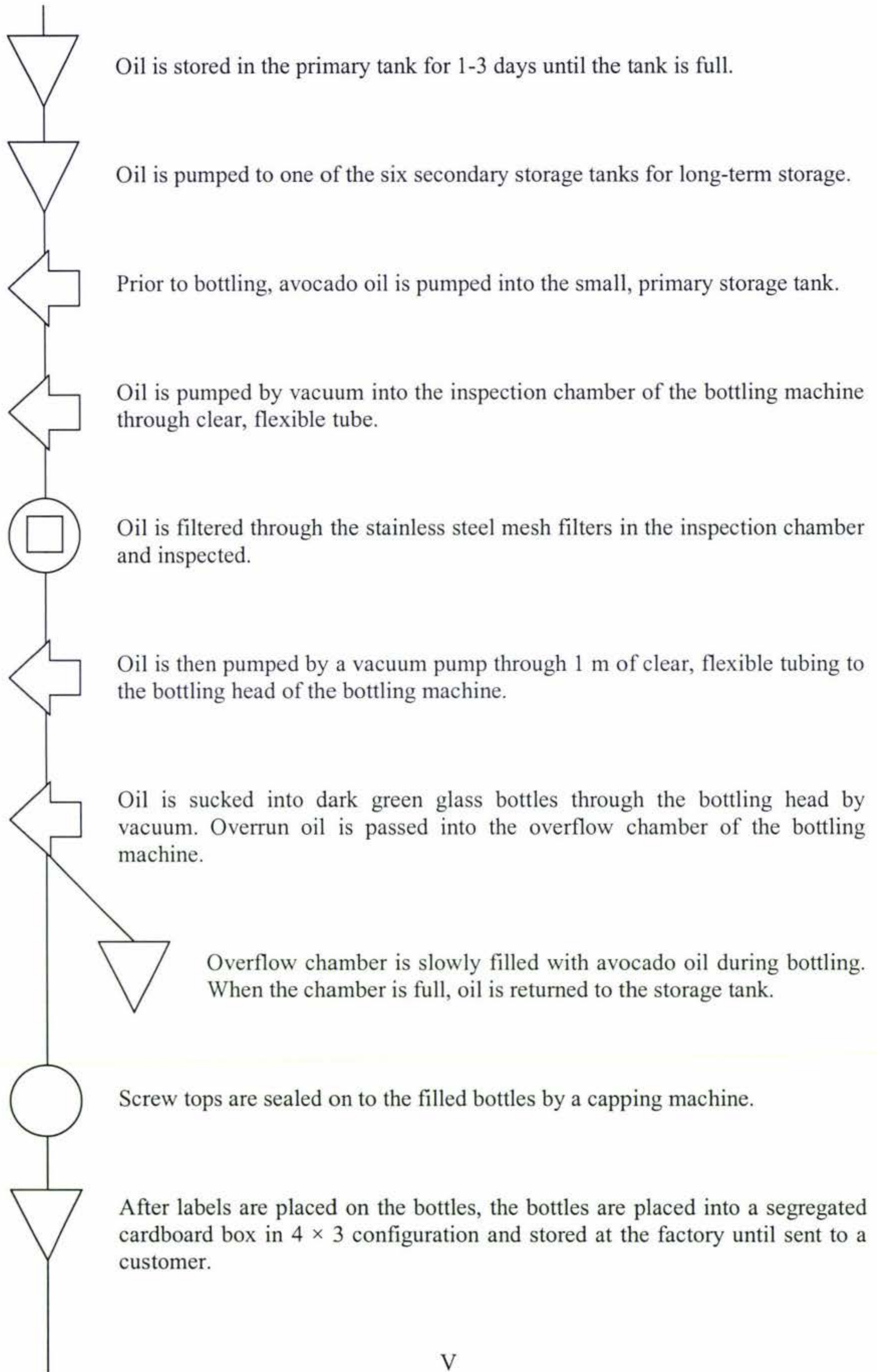


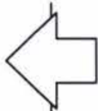
Fruit is heated and stirred again in the secondary malaxers for about an hour.


Pulp is pumped by mono pump through 6m of clear 55mm ID flexible hosing to the decanter.
Water is added to the fruit pulp just prior to separation in the decanter to allow better separation.
The three phase decanter separates the pulp into 3 phases; a heavy phase consisting mostly of water, a light phase consisting mostly of oil and a solid phase consisting of exhausted pulp and skin and stone fragments.
The solid phase is pumped out of the building by a large auger and exits the process.
The light phase passes through a short section of pipe to the light phase collection tray.
The heavy phase passes through a short section of stainless steel pipe to the heavy phase collection tray.
The light phase collects in a stainless steel tray beneath the decanter.
The heavy phase collects in a separate stainless steel tray beside the light phase tray.
The light phase is continuously pumped from its tray through 12 m of clear and flexible line to the light phase header tank above the oil centrifuge.
The heavy phase is continuously pumped from its tray through 12m of clear and flexible line to the heavy phase header tank above the water centrifuge.

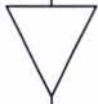


The light phase remains in the header tank for a short period until it is manually regulated into the oil centrifuge.
The heavy phase, remains in the header tank and is manually regulated into the water centrifuge.
Clean water is added to the light phase during entry into the oil centrifuge to aid separation of pure oil.
Water is added to the heavy phase during entry into the water centrifuge to aid separation of any oil remaining in the heavy phase.
Water and other contaminants are removed from the light phase during centrifugation and exit the process. Pure oil is passed into the mass flowmeter.
Oil is removed from the water and other contaminants in the heavy phase by the water centrifuge. The oil is returned to the light phase collection tray beneath the decanter for further separation.
Oil collects in the 105 litre reservoir of the mass flowmeter where it can remain for a long period of time since the reservoir is not continuously displaced.
Oil passes into the weighing funnel of the mass flowmeter from the initial reservoir where it is batch weighed, then dumped into the second holding tank of the mass flowmeter.
Oil remains in the secondary reservoir which is much smaller in volume than the primary reservoir, until it is pumped out by a float-switch controlled pump.
Oil is then pumped through a solid stainless steel line to the primary storage tank.

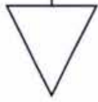

Oil is stored in the primary tank for 1-3 days until the tank is full.
Oil is pumped to one of the six secondary storage tanks for long-term storage.
Prior to bottling, avocado oil is pumped into the small, primary storage tank.
Oil is pumped by vacuum into the inspection chamber of the bottling machine through clear, flexible tube.
Oil is filtered through the stainless steel mesh filters in the inspection chamber and inspected.
Oil is then pumped by a vacuum pump through 1 m of clear, flexible tubing to the bottling head of the bottling machine.
Oil is sucked into dark green glass bottles through the bottling head by vacuum. Overrun oil is passed into the overflow chamber of the bottling machine.
Overflow chamber is slowly filled with avocado oil during bottling. When the chamber is full, oil is returned to the storage tank.
Screw tops are sealed on to the filled bottles by a capping machine.
After labels are placed on the bottles, the bottles are placed into a segregated cardboard box in 4 × 3 configuration and stored at the factory until sent to a customer.



Boxes are stacked onto pallets and sent to customers.



Retailer display oil bottles on well lit shelves for up to 12 months prior to purchase by a consumer.



Consumer opens bottle and may store in light/dark conditions for up to 3-6 months during use.

11.2 Q_{10} and predicted shelf life calculation for 1 month old EVAO

$$
\begin{aligned}
Q_{10\,(1\text{ month old EVAO})} &= \frac{330}{275} \\
&= 1.20
\end{aligned}
$$

$$
\begin{aligned}
\log t_s &= \frac{\log 1.20}{10} \times 25 + \left(\frac{\log 1.20}{10} \times 50 + \log 330 \right) \\
&= 3.112 \\
&\Rightarrow t_s = 1295\,\text{hours} \\
&\sim 54\,\text{days}
\end{aligned}
$$

11.3 Q_{10} and predicted shelf life calculation for 10 month old EVAO

$$
\begin{aligned}
Q_{10\,(10\text{ month old EVAO})} &= \frac{180\text{ hours}}{210\text{ hours}} \\
&= 0.86
\end{aligned}
$$

$$
\begin{aligned}
\log t_s &= \frac{\log 0.86}{10} \times 25 + \left(\frac{\log 0.86}{10} \times 50 + \log 180 \right) \\
&= 1.76 \\
&\Rightarrow t_s = 58\,\text{hours} \\
&\sim 2\,\text{days}
\end{aligned}
$$

11.4 Q_{10} and predicted shelf life calculation for 4 month old RBD avocado oil

$$Q_{10\,(4\ \text{month old RBD})} = \frac{105\ \text{hours}}{105\ \text{hours}} = 1.00$$

$$\log t_s = \frac{\log 1.00}{10} \times 25 + \left(\frac{\log 1.00}{10} \times 50 + \log 105 \right)$$

$$= 2.02$$

$$\Rightarrow t_s = 105\ \text{hours}$$

$$\sim 4.5\ \text{days}$$