Comparison of Chemical Quality Standards for New Zealand Extra Virgin Olive Oil

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Abstract

Extra virgin olive oil (EVOO) is the highest quality and most expensive olive oil classification. There are three international quality standards (IOC, USDA and Standards Australia) for defining the chemistry and sensory quality of EVOO. The objectives of the present work were to analysis New Zealand EVOO according to specific standards applied in Australia which are different from IOC and USDA standards, predict the shelf life of New Zealand EVOO based on the new standard tests and evaluate how commercial EVOOs available in New Zealand fit with the Australia standard based on chemical analysis only. Two brands of avocado oil were taken into consideration in the commercial oils comparison, tested with the same chemical analyses as for the EVOOs. EVOO from Waiheke Island, New Zealand (NZ), was stored and evaluated for various quality standards over a six-month storage trial. EVOO from two cultivars, 'Frantoio' and 'Leccino', was analysed. The storage temperatures selected were 7, 20, 30 and 40 °C. The peroxide value, pyropheophytin% and 1,2-diacylglycerols% were analyzed using titration, HPLC and GC, respectively, based on the methods outlined in the olive oil standards. Zero and first order kinetics was applied to the data collected for peroxide value, pyropheophytin% and 1,2-diacylglycerols% in order to predict the EVOO shelf life. The commercial EVOOs available in NZ were compared by analyzing the % free fatty acid, peroxide value, total phenolics, pyropheophytin% and 1,2-diacylglycerols%. The commercial EVOOs from NZ had lower pyropheophytin% and higher 1,2-diacylglycerols% than imported olive oils. Commercial avocado oils tested showed high % free fatty acid and low total phenolics, low pyropheophytin% but low 1,2-diacylglycerols% compared to NZ olive oil.

A high content of phenolic compounds and of 1,2-diacylglycerols% were found in New Zealand EVOO which was the best oil compared to other countries' EVOO.

New Zealand EVOO can be stored for at least one year at room temperature (20 °C) and still remain within the Australian Standards.

Key words:

extra virgin olive oil (EVOO), New Zealand, standards, pyropheophytin, 1,2-diacylglycerols.

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Chapter One: Introduction

Extra virgin olive oil (EVOO) without any oxidization is full of natural antioxidants such as polyphenols, vitamin E and squalene (Frankel, 2011). The importance of EVOO is mainly attributed to its high content of oleic acid and its richness in phenolic compounds, which act as natural antioxidants (Lerma-García et al., 2010). EVOO is expensive owing to the hard and time-consuming tasks involved in the cultivation of olive trees, the harvesting of the fruits, and the extraction of the oil (Lerma-García et al., 2008). For these reasons, changes in the quality of EVOO during storage are important to monitor.

To trace the quality changes of EVOO, a number of chromatographic and spectrophotometric techniques have been used in this research, such as HPLC and GC will be applied to detect changes in the chemical composition of EVOO during the storage. Based on the trends of the chemical changes during storage of EVOO, the prediction of shelf life will be obtained.

There are three international quality standards (IOC, USDA and Standards Australia) for defining the chemistry and sensory quality of EVOO (International Olive Council, 2010a; United States Standards for Grades of Olive Oil and Olive-Pomace Oil, 2010; Standards Australia, 2011). For the commercial EVOO, industries aim to ensure EVOO stability until the end of the best before date for consumers (Pagliarini, Zanoni & Giovanelli, 2000). Good olive oil extraction procedures can ensure good oil quality with low levels of oxidation and oil oxidation can be reduced if oils are stored in appropriate containers (Boskou, 1996d). Oils oxidation occurs spontaneously, therefore they will deteriorate after long storage times (Boskou, 1996d).

The aim of the present research is to analyse New Zealand EVOO according to specific standards applied in Australia which are different from IOC and USDA standards (International Olive Council, 2010a; United States Standards for Grades of Olive Oil and Olive-Pomace Oil, 2010; Standards Australia, 2011). Furthermore, the study has been conducted with the use of different storage conditions to set up a predictive model of stability in order to predict the shelf life of New Zealand EVOO. Finally, based on the new standards, tests were carried out to evaluate how commercial EVOOs available in New Zealand fit with the various standards. In addition, two brands of avocado oil were included in the commercial oils for comparison.

Chapter Two: Literature Review

2.1 Olive fruit composition

The olive fruit composition varies with different cultivars, the environment and the degree of ripeness. An average chemical composition (%) of olive fruit is shown in Table 1 (Kiritsakis, 1990).

Table 1. Chemical composition of olive fruit

Component	Percent
Water	50.0
Oil	22.0
Sugars	19.1
Cellulose	5.8
Protein	1.6
Ash	1.5
TOTAL	100

2.2 Olive oil definition and classification

Olive oil is the oily juice of the olive fruit and it mainly exists in the pulp of the stone fruit of the olive tree (*Olea europaea sativa*) (Belitz et al., 2009). Olive oil has been divided into two main categories. The first category is olive oil which includes virgin olive oil, refined olive oil and olive oil. For the olive oil, it can be a blend of virgin olive oil and refined olive oil (International Olive Council, 2010a). These oils are all obtained directly from the olive fruit without the use of solvents or re-esterification.

The second category is olive-pomace oil which is obtained by treating olive pomace with solvents or other physical treatments (excluding re-esterification processes) (International Olive Council, 2010a; United States Standards for Grades of Olive Oil and Olive-Pomace Oil, 2010; Standards Australia, 2011).

Virgin olive oil has three types: extra virgin olive oil, fine virgin olive oil and semi-fine virgin olive oil (or ordinary virgin olive oil) (Boskou, 1996a). Extra virgin olive oil (EVOO) is the highest olive oil quality grade (Frankel et al., 2010; Laddomada et al., 2013). This type of virgin olive oil has the best flavour and odour, and a free acidity percentage which is expressed as oleic acid, of not more than 0.8 grams per 100 grams. (Boskou, 1996b; Luchetti, 2000; Bianchi, 2002; International Olive Council, 2010a; United States Standards for Grades of Olive Oil and Olive-Pomace Oil, 2010; Standards Australia, 2011) .

2.3 Composition of olive oil

Olive oil consists of primarily triacylglycerols (approximately 99%) and secondly free fatty acids (FFA) whose proportion depends on the extent of triglyceride hydrolysis, mono- and diacylglycerols and an array of non-saponifiable compounds and pigments (Kiritsakis, 1998; Boskou et al., 2006a). The composition of fatty acid varies according to the variety of olive oil, climatic conditions, production location and the stage of maturity of the fruit (Harwood & Aparicio, 2000; Boskou et al., 2006a). The components of extra virgin olive oil are divided into two groups according to their concentration: major and minor compounds (Table 2). The first group, known as the saponifiable fraction, represents > 98% of the total weight of the oil and consists of triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids and phosphatides. The second group, the unsaponifiable fraction, comprises about 2% of the total weight, it includes fatty alcohols, sterols, hydrocarbons, volatile compounds, and antioxidants, including carotenoids, chlorophyll, tocopherols and phenols. (Kiritsakis, 1990;

Wiesman, 2009; Medina et al., 2011). The colour of olive oil is determined by the concentration of pigments in the original fruits. The best resulting oil is defined as green-yellowish although the green hue may vary according to some factors such as variety of fruit and the ripeness of the fruits used (Gandul-Rojas et al., 2000; Roca & Mosquera, 2001).

Table 2. The components of extra virgin olive oil.

Fraction	Percentage of total weight	compounds
saponifiable fraction	about 98%	triacylglycerols diacylglycerols monoacylglycerols free fatty acids
unsaponifiable fraction	about 2%	fatty alcohols sterols hydrocarbons volatile compounds antioxidants (carotenoids , chlorophyll, tocopherols and phenols)

2.4. How to extract olive oil

There are four main steps used to extract olive oil:

2.4.1. Fruit cleaning (defoliation, olive washing): leaf removal and washing.

2.4.2. Preparation of the paste (crushing, malaxing): crushing is carried out to tear olive fruits cells in order to release the droplets of oil; malaxing breaks up the oil/water emulsion, so that the droplets of olive oil can join together to form larger droplets (Boskou et al., 2006a).

2.4.3. Separation of the solid (pomace) and liquid phases (oily must and wastewater). There are three options for mechanical methods to separate solid and liquid phases:

a. First method is the pressing method (Figure 1) which is used to obtain virgin olive oil under appropriate conditions using a hydraulic press and mats. This method guarantees top quality oil as it maintains low temperatures throughout the entire operation. However, it requires more labour and it is not a continuous operation (Boskou et al., 2006a).

b. Second method is the centrifugation method (Figure 2) using a decanter which operates by using centrifugal separation of the liquid from the olive paste. It is continuous-operation, costs for labour are less and has high processing capacity. There are two types of centrifuge systems available, three-phase and two-phase centrifugation:

Two ways of centrifuging:
Three-phase Centrifugation:
In order to facilitate separation of the fractions with different specific weights, lukewarm water is added. The water-thinned paste is centrifuged in the decanter. Three phases are obtained: an oily must, vegetable water mixed with the added water (OMWW), and olive pomace (stones and pulp residue). Disadvantages of a three-phase system are increased water utilization, loss of valuable components (e.g. polyphenols) in the water phase and the problems with the waste water disposal. The polyphenols are important substances in virgin olive oil, but are more soluble in water

than in oil. Hence, water needs to be recycled during this process in order to reduce the water usage (Giovacchino, 2000).

Two-phase Centrifugation:

Two-phase centrifugation uses no added water and delivers oil as the liquid phase, and produces wet olive pomace (moisture content 60 ± 5%). The disrupted paste is centrifuged in the decanter and then the two phases are obtained; the oily must and a solid/water mixture (pomace) (Boskou et al., 2006a).

Compared to three-phase centrifugation, the two-phase process produces similar yields of olive oil but of a superior quality in terms of polyphenols and o-diphenols content and storage stability. Less waste water is produced during oil extraction and there is also reduced requirement of water but it creates a high moisture content pomace which is difficult to handle (Boskou et al., 2006a).

c. Third method is the percolation method (Figure 3). It takes place at ambient temperature, does not need additional dilution water and does not use mats, thus any possibility of contamination is avoided. In addition, it is an automated process that needs a small workforce and a limited amount of energy, but it cannot provide high oil yields. This method is used in combination with the centrifugal decanter in order to make the operation continuous and increase oil yields. The combined process (Figure 4) produces a similar yield as the pressing method, but it is continuous, which reduces cost and increases capacity.

2.4.4. Separation of the liquid phases (oil/wastewater) using centrifugation or decantation (Giovacchino, 2000; Boskou et al., 2006a).

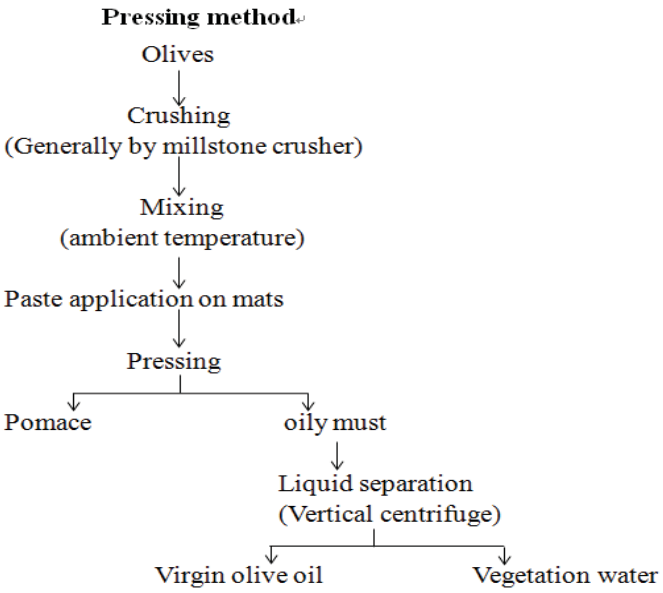


Figure 1. Diagram of olive oil extraction by pressing method (Giovacchino, 2000).

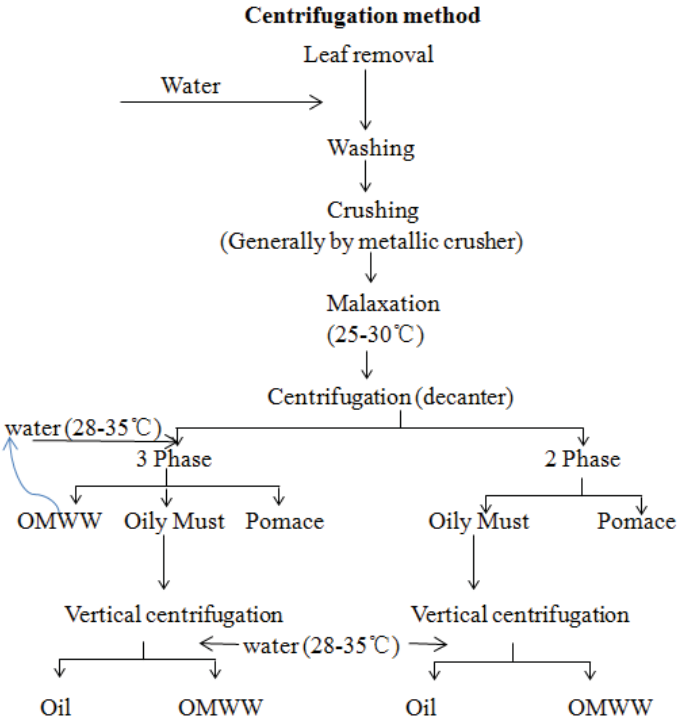


Figure 2. Extraction by the centrifugation method (Boskou et al, 2006a)

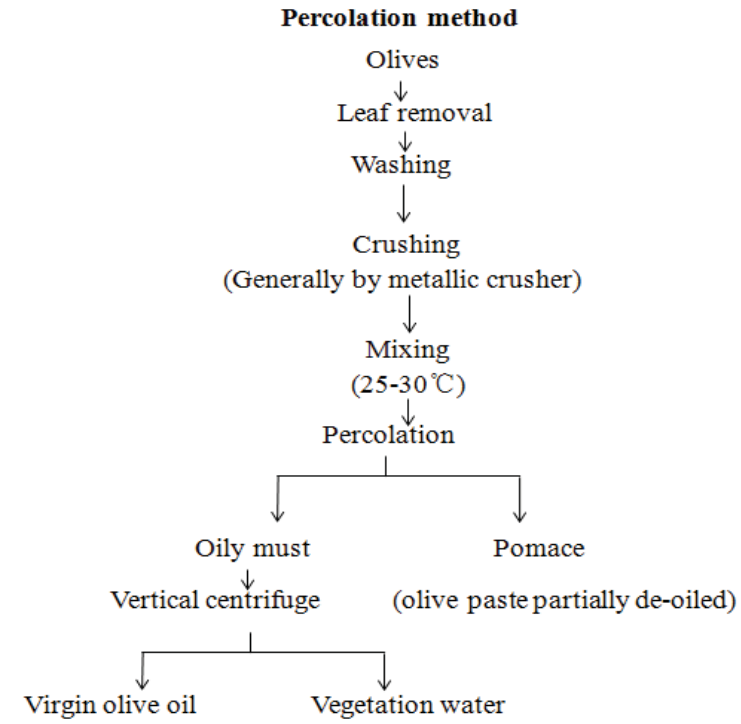


Figure 3. Diagram of olive oil extraction by percolation method (Giovacchino, 2000).

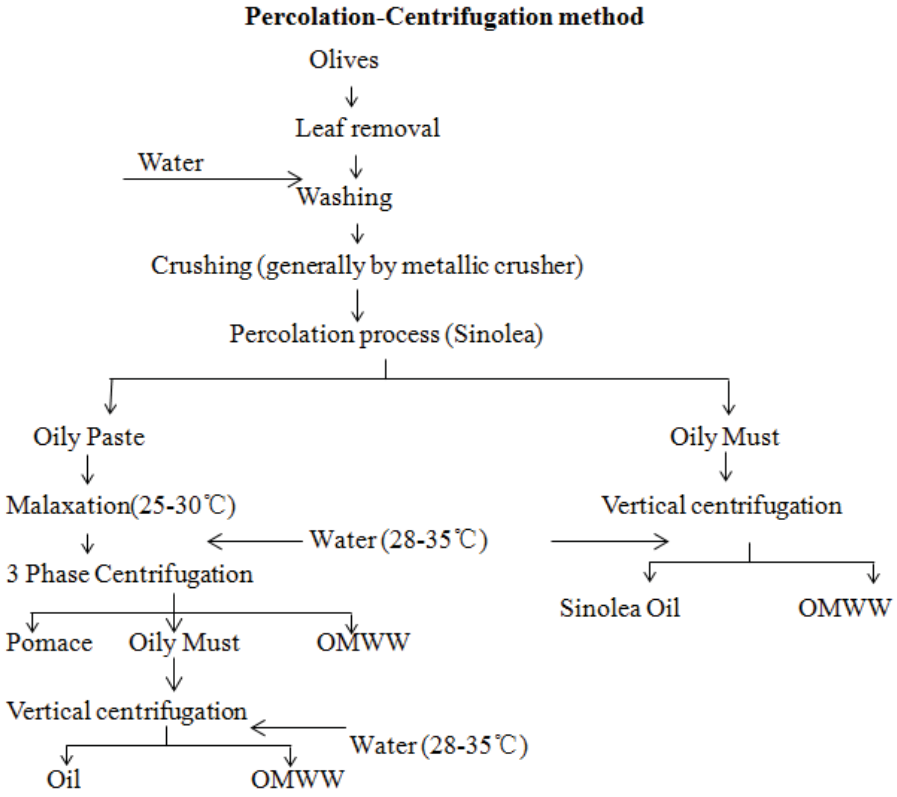


Figure 4. Extraction by percolation-centrifugation method (Boskou et al., 2006a).

The modern means to extract virgin olive oil is by centrifugation systems because it produces high-quality oils with less production loss (Garcia-Gonzalez & Aparicio, 2010).

2.5 The necessity of a standard for olive oil.

Standards for olive oil cover the oil's quality and purity. The purity standards for olive oil are to ensure the absence of adulterants, as olive oil is a perfect target for defrauders due to its high price and reputation (Garcia-Gonzalez & Aparicio, 2010). Instrumental advances are necessary in order to fight against adulteration (García-González & Aparicio, 2010). There are many media reports of fraud in the olive oil business, where extra virgin olive oils have been adulterated with cheaper seed oils such as hazelnut, sunflower and soybean or refined olive oils (Gurdeniz & Ozen, 2009; Frankel et al., 2010; Torrecilla et al., 2011; Garcia et al., 2013). These adulterations are difficult to detect, especially when their concentrations are less than 10 % (Torrecilla et al., 2011). In the meantime the perpetrators have also advanced their technology in order to adulterate. Therefore, the pace of fighting against adulteration activities needs to move rapidly to counteract fraudulent practises (Garcia-Gonzalez &Aparicio, 2010). Hence, standards need to be checked, monitored and if necessary improved continually (Tsimidou, 2006). The European Union (EU) has unified the analytical methods for detecting the authenticity of virgin olive oil (Sacchi et al., 1997). At the same time, the efforts of developing new analytical methods are continuing in the EU (Sacchi et al., 1997). The International Olive Oil Council (International Olive Council, 2012) and the Commission regulation (EC, 1991) have classified the quality of olive oil based on parameters that include free fatty acid (FFA) content, peroxide value (PV), UV specific extinction coefficients (K_{232} and K_{270}) and sensory score (Kalua et al., 2007).

2.6 Factors affecting the quality of olive oil

Virgin olive oil quality depends on many factors related to olive tree cultivation, the quality of fruits, the harvesting time of the olives, storage and the olive processing steps such as the techniques utilized to extract the oil, especially the machines for crushing the olives, for the kneading of the olive paste and separation of the oil phase (Giovacchino, 2000; Di Vincenzo, 2002; Amirante et al., 2006; Mailer, 2007). The moment of picking is a decisive factor to ensuring good quality of the olive oil as well (Di Vincenzo, 2002). Exposure to oxygen, light and heat is detrimental to the quality of the oil which leads to a consequence of oxidative and hydrolytic degradation reactions (Méndez & Falqué, 2007). The storage and packing conditions are also going to determine the commercial life of the olive oil (Méndez & Falqué, 2007).

2.7 The reason for the appearance of USA and standards Australia for olive oil

The International Olive Council (IOC) is the only international intergovernmental organization of the world in the field of olive oil and table olives (Frankel et al., 2010). It was established in Madrid, Spain, in 1959, under the auspices of the United Nations (Frankel et al., 2010). Originally known as the International Olive Oil Council or IOOC, in 2006 they changed their name to the International Olive Council to include table olives and not just olive oil (International Olive Council, 2012). The United States is not a member of the IOC and has had their own olive oil standards since 1948 (Frankel et al., 2010; USDA Standards, 2010), which was earlier than the establishment of the International Olive Oil Council (IOOC).

Australia has planted a wide range of olive cultivars all producing olive oil with varying composition (Mailer, 2007). Sometimes the composition results fall outside the IOC purity standards (Mailer, 2007; Mailer, 2012). The IOC standards have very

tight limits for some components, which leads to some good quality olive oils produced in Australia not meeting the IOC criteria. As a result, a new standard for olive oil that is more suitable to the conditions of Australia was needed (Mailer, 2007). In 2011, Australia published the Australian Standard: Olive oils and olive-pomace oils (Standards Australia, 2011).

2.8 The difference between the different olive oil standards

In terms of the quality criteria, compared to the IOC Standard, the USA Standards added odour and taste, colour, halogenated solvents, heavy metals such as lead (Pb) and arsenic (As), and pesticide residues (International Olive Council, 2010a; United States Standards for Grades of Olive Oil and Olive-Pomace Oil, 2010). The USA Standards do not include the concentration of fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs). Australia's quality criteria standards are similar to the IOC standards except they have added quality standards for pyropheophytin a (PPPs) and 1,2-diacylglycerols (DAGs) and deleted unsaponifiable matter (Standards Australia, 2011). In the quality criteria for virgin olive oil, only the "median of defects" is different among the three countries. The standards for quality criteria of extra virgin and virgin olive oil from the IOC, USA and Australia are presented in Tables 3, 4 and 5. Any differences across the standards are highlighted in yellow. Codex Alimentarus's standard for olive oils and olive pomace oils is in line with the IOC standards.

Table 3. Quality Criteria (extra virgin olive oil).

		IOC	USA	AUS
1.Organoleptic characteristics				
-odour and taste		ND	Excellent	ND
-odour and taste (on a continuous scale):		Median=0	Median=0	Median=0.0
.median of defect				
.median of the fruity attribute		Median>0	Median>0	Median>0.0
Colour		ND	yellow to green	ND
aspect at 20°C for 24 hours		ND	ND	ND
2.Free fatty acid content (FFA) (%m/m)		≤0.8	≤0.8	≤0.8
3.Peroxide Value, In Milleq. Peroxide Oxygen Per kg/oil		≤20	≤20.0	≤20
4.Absorbency in ultra-violet(K% 1cm)	270 nm	≤0.22	≤0.22	≤0.22
	ΔK	≤0.01	≤0.01	≤/0.01/
	232 nm	≤2.50	≤2.50	≤2.50
5.Moisture and volatile matter (% m/m)		≤0.2	≤0.2	≤0.2
6.Insoluble impurities in light petroleum % m/m		≤0.1	≤0.1	≤0.1
7.Flash point		ND	ND	ND
8.Trace metals(mg/kg)	Iron	≤3.0	≤3.0	≤3.0
	Copper	≤0.1	≤0.1	≤0.1
9. Fatty acid methyl esters (FAMEs) and fatty acid ethyl		-∑FAME+FAEE≤75mg/kg or	ND	ND

esters (FAEEs)	-∑FAME+FAEE>75mg/kg and ≤150mg/kg And FAEE/FAME ratio≤1.5			
10.Halogenated Solvents	ND		Maximum content of each halogenated solvent 0.1mg/kg	ND
11.Heavy Metals Lead (Pb)			Maximum content of all halogenated solvents 0.2 mg/kg	ND
Arsenic (As)	ND			
12. Pesticide Residues	ND		The products covered by this standard shall comply with the maximum residue limits established by the U.S. Environmental Protection Agency.	ND
13.Pyropheophytin a (PPPs)(%)		ND	ND	≤17
14.1,2-Diacylglycerols (DAGs)(%)		ND	ND	≥35

(Boskou,1996b; Kiritsakis, 1998; Harwood and Aparicio, 2000; International Olive Council, 2010a; United States Standards for Grades of Olive Oil and Olive-Pomace Oil, 2010; Standards Australia, 2011).

ND: Not detectable.

Bold Yellow Block: the difference among IOC, USA and Standards Australia.

Table 4 Quality Criteria (virgin olive oil)

		IOC	USA	AUS
1.Organoleptic characteristics		ND	Good	ND
-odour and taste				
-odour and taste (on a continuous scale):		0<Median<3.5	0<Median≤2.5	0.0<Median<2.5
.median of defect				
.median of the fruity attribute		Median>0	Median>0	>0.0
Colour		ND	yellow to green	ND
aspect at 20℃ for 24 hours		ND	ND	ND
2.Free fatty acid content (FFA) (%m/m)		≤2.0	≤2.0	≤2.0
3.Peroxide Value, In Milleq. Peroxide Oxygen Per kg/oil		≤20	≤20.0	≤20
4.Absorbency in ultra-violet(K% 1cm)	270 nm	≤0.25	≤0.25	≤0.25
	ΔK	≤0.01	≤0.01	≤/0.01/
	232 nm	≤2.60	≤2.60	≤2.60
5.Moisture and volatile matter (% m/m)		≤0.2	≤0.2	≤0.2
6.Insoluble impurities in light petroleum % m/m		≤0.1	≤0.1	≤0.1
7.Flash point		ND	ND	ND
8.Trace metals(mg/kg)	Iron	≤3.0	≤3.0	≤3.0
	Copper	≤0.1	≤0.1	≤0.1
9. Fatty acid methyl esters (FAMEs) and fatty acid ethyl		ND	ND	ND

esters (FAEEs)			
10.Halogenated Solvents	ND	Maximum content of each halogenated solvent 0.1mg/kg	ND
11.Heavy Metals Lead (Pb) Arsenic (As)	ND	Maximum content of all halogenated solvents 0.2 mg/kg	ND
12. Pesticide Residues	ND	The products covered by this standard shall comply with the maximum residue limits established by the U.S. Environmental Protection Agency.	ND
13.Pyropheophytin a (PPPs)(%)	ND	ND	ND
14.1,2-Diacylglycerols (DAGs)(%)	ND	ND	ND

(Boskou,1996b; Kiritsakis, 1998; Harwood and Aparicio, 2000; International Olive Council, 2010a; United States Standards for Grades of Olive Oil and Olive-Pomace Oil, 2010; Standards Australia, 2011)

ND: Not detectable.

Bold Yellow Block: the difference among IOC, USA and Standards Australia.

Table 5 Purity Criteria (Edible natural olive oils)

			IOC (Edible virgin olive oils; Virgin olive oils)	USA (US Extra Virgin Olive Oil; US Virgin Olive Oil)	AUS (Edible natural olive oils)
1.Fatty acid composition as determined by gas chromatography (% m/m methyl esters):		Myristic acid(C14:0)	≤0.05	≤0.05	≤0.05
		Palmitic acid (C 16:0)	7.5-20.0	7.5-20.0	7.0-20.0
		Palmitoleic acid (C 16:1)	0.3-3.5	0.3-3.5	0.3-3.5
		Heptadecanoic acid (C17:0)	≤0.3	≤0.3	≤0.3
		Heptadecanoic acid (C17:1)	≤0.3	≤0.3	≤0.4
		Stearic acid (C18:0)	0.5-5.0	0.5-5.0	0.5-5.0
		Oleic acid (C18:1)	55.0-83.0	55.0-83.0	53.0-85.0
		Linoleic acid (C18:2)	3.5-21.0	3.5-21.0	2.5-22.0
		Linolenic acid (C18:3)	≤1.0	≤1.5	≤1.5
		Arachidic acid (C20:0)	≤0.6	≤0.6	≤0.6
		Gadoleic acid (eicosenoic) (C20:1)	≤0.4	≤0.4	≤0.5
		Behenic acid (C22:0)	≤0.2	≤0.2	≤0.2
		Lignoceric acid (C24:0)	≤0.2	≤0.2	≤0.2
2.Trans Fatty Acid Content (% trans fatty acids)		C18:1T %	≤0.05	≤0.05	≤0.05
		C18:2 T+C18:3 T%	≤0.05	≤0.05	≤0.05
3.Sterol and triterpene dialcohol	Desmethylsterol composition (%)	Cholesterol	≤0.5	≤0.5	≤0.5
		Brassicasterol	≤0.1	≤0.1	≤0.1

composition :	total sterols)	Campesterol	≤4.0		≤4.5		≤4.8
		Stigmasterol	< campesterol in edible oils		< campesterol in edible oils		≤1.9
		Delta-7-stigmastenol	≤0.5		≤0.5		≤0.5
		Apparent beta-sitosterol: Clerosterol +Sitostanol +Beta-Sitosterol+ Delta 5-24-Stigmastadienol+ 5-23-Stigmastadienol+ Delta-5-Avenasterol		≥93.0		≥93.0	≥92.5
	Total sterol content (mg/kg)		≥1000		≥1000		≥1000
	Erythrodiol and uvaol content (% total sterols)		≤4.5		≤4.5		≤4.5
4. Wax content C40+C42+C44+C46 (mg/kg)			≤250		≤250		≤250
5. Maximum difference between the actual and theoretical ECN 42 triacylglycerol content			0.2		|≤0.2|		≤/0.2/
6. Stigmastadiene content (mg/kg)			≤0.10		≤0.15		≤0.10
7.Content of 2-glyceryl monopalmitate			C16:0≤14 % C16:0>14 %	2P≤0.9 % 2P≤1.0 %	C16:0≤14 % C16:0>14 %	2P≤0.9 % 2P≤1.0 %	≤1.5%
8. Unsaponifiable matter (g/kg)			≤15		≤15		ND

(Boskou,1996b; Kiritsakis, 1998; Harwood and Aparicio,2000; International Olive Council, 2010a; United States Standards for Grades of Olive Oil and Olive-Pomace Oil, 2010; Standards Australia, 2011)

ND: Not detectable.

Bold Yellow Block: the difference among IOC, USA and Standards Australia.

2.9 Olive oil colour

Oil colour is one of the basic quality characteristics of virgin olive oil (Minguez-Mosquera et al., 1991). The pigments presenting in the original fruit gives the specific colour of olive oil. The product is best defined by a green-yellowish colour (Gandul-Rojas et al., 2000). At first, the method of evaluating of colour of olive oil is using the visual comparison with a standard solution (Gandul-Rojas et al., 2000). Currently, colorimeters have been adopted, which automatically carry out this process. However, the instruments of colorimetric methods without green or blue hues are not appropriately suitable for olive oil. The colour in olive oil is due to the presence of chlorophyll and cartenoid pigments. To analyse these pigments in olive oil, the high-performance liquid chromatography (HPLC) can be used in the separation and quantification of chlorophyll and carotenoids (Gandul-Rojas et al., 2000).

2.9.1 Chlorophyll definition

The green colour in olive oil is due to the presence of chlorophyll. Chemically chlorophyll is a mixture of several highly complex molecules, which consist of a ring structure (the porphyrin structure) with a central magnesium ion, and a long hydrophobic side chain (Figure 5).

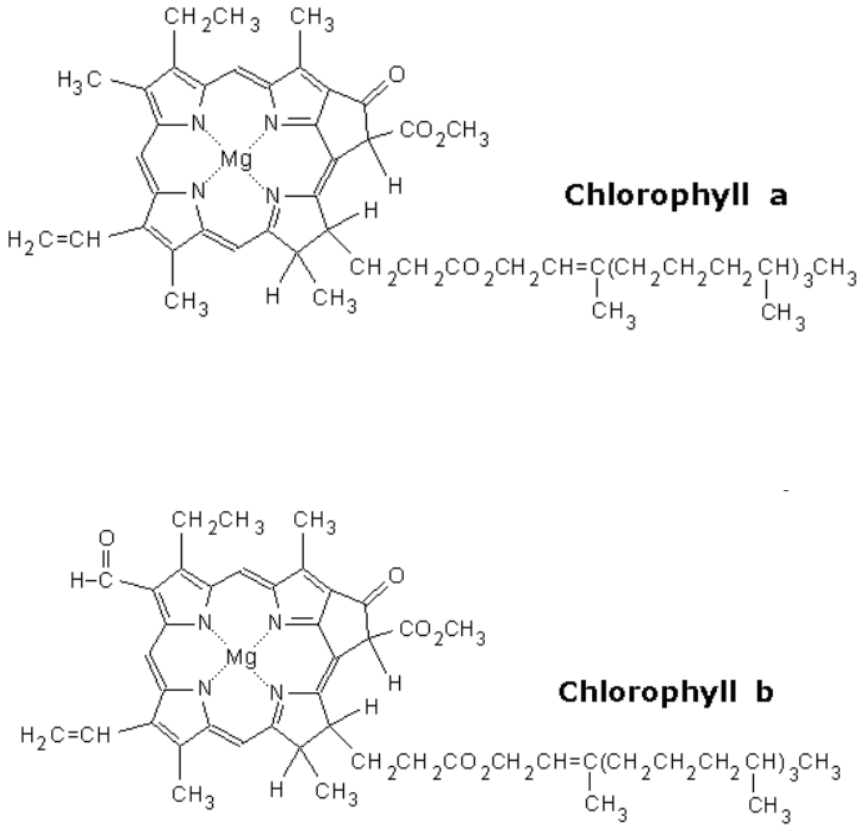


Figure 5. Structure of the two most common chlorophylls (sourced from food-info.net) (2011). Available at: http://www.food-info.net/uk/colour/chlorophyll.htm

Olive oil contains the pigments chlorophyll a and chlorophyll b (Figure 5) which are green and pheophytin a and pheophytin b which are brown (Kiritsakis, 1991). Chlorophylls (chlorophyll a and b) are the pigments that attribute to the green colour of the olive drupe (Olea europea L.) when it begins to ripen (Giuliani et al., 2011). Hence, changes in the chlorophyll fraction profile affects both olives and the oil extracted from them (Roca & Miguea-Mosquera, 2001; Giuliani et al., 2011). It has been found that there are higher chlorophyll values in the oils obtained by centrifugation than by pressure extraction (Kiritsakis, 1991). Chlorophylls and carotenoids are pigments that can colour olives and olive oil to be green and yellow (Minguez-Mosquera, et al., 1990). They are also the indicators of the quality of

finished products (Minguez-Mosquera, et al., 1990; Gandul-Rojas et al., 2000). Gandul-Rojas et al. (2000) reported that the presence of a specific pigment profile could become a requirement of virgin olive oil, and the ratio between pigments could guarantee the authenticity of the product. A number of researchers have reported that using the pigment profile as an indicator of authenticity would help prevent the marketing of fraudulent mixtures with vegetable oils from another source and also it is proposed as a new index of quality of virgin olive oil (Minguez-Mosquera et al., 1992; Gandul-Rojas et al., 2000; Roca & Mosquera, 2001).

2.9.2 The formation of pyropheophytin and detection

The colour of olives changes from bright green to olive brown during processing and storage due to the degradation of chlorophyll (Figure 6). The degradation occurs in two ways: loss of Mg due to heat and/or acid substitution of Mg^{2+} by H^{+} (possibly also enzymatically) or loss of the phytol group through the action of the enzyme chlorophyllase (Giuffrida et al., 2007). As for the formation of pyropheophytin, the "pyro" derivatives are formed from the corresponding pheophytins by the loss of the carbomethoxy group. The degradation of chlorophyll in olives is presumably a combination of enzymatic activity and chemical changes (Schwartz & Elbe, 1983; Boekel, 2000).

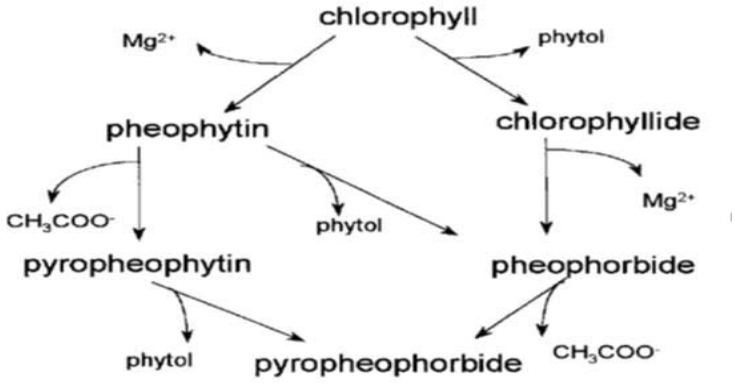


Figure 6. Schematic representation of pathways of chlorophyll degradation (Boekel, 2000)

Chlorophyll pigments break down to pheophytins and then pyropheophytins upon thermal degradation of olive oil. An elevated level of pyropheophytins is an indicator for oil that has oxidized and/or been adulterated with refined oil (Frankel et al., 2010). Heating is required in refining olive oil, as a consequence this leads to chlorophyll converting into pyropheophytin a (Mailer, 2007). The difference between refined and EVOO is in the refining process which leads to the bleaching of the oil and hence the total disappearance of the natural chlorophyll pigments. The green colour of such oils is often obtained by illegally adding synthetic chlorophyll pigment (Giuliani et al., 2011).

There is oil refining technology available which allows virgin olive oil (VOO) deodorization to be conducted at a moderate temperature ($\leq$ 100 °C), which can effectively remove volatiles that are responsible for undesirable attributes (García-González & Aparicio, 2010). Labelling this oil as VOO is banned because VOO cannot be exposed to high temperatures, but if the VOO was to be deodorized at low temperatures it cannot be detected (Garcia-Gonzalez & Aparicio, 2010). The ratio between pheophytin a (a natural chlorophyll) and pyropheophytin a (a thermal degradation compound) has been used by some researchers to detect this form of adulteration (García-González & Aparicio, 2010). The acid compounds liberated from the fruits during the oil extraction process promote the production of pheophytin from chlorophyll degradation (Gallardo-Guerrero et al., 2005). During the first three months of storage, the amount of pheophytin in the oil will increase (Gallardo-Guerrero et al., 2005). At the same time, isomerized xanthophylls (a carotenoid) and allomerized pheophytins increase slightly. Following this stage, pyropheophytin a (a pigment not present in the initial oils), can be detected and its concentration increases during storage (Gallardo-Guerrero et al., 2005). The amount of pyropheophytin a increases during the shelf life of a VOO but varies from one oil to another depending on the initial pheophytin amount, which depends on many factors such as ripening stage of fruits, cultivar and seasonal conditions etc. However,

the ratio of pheophytin a/total pyropheophytin is independent on these factors and may be used as an indicator of the presence of adulteration (Hornero-Méndez et al., 2005; Garcia-Gonzalez & Aparicio, 2010; Garcia et al., 2013).

The degradation products pheophytin a, a' and pyropheophytin a of chlorophylls can be analysed by reverse phase high performance liquid chromatography (HPLC). A miniaturized column chromatography on a silica gel column is used to separate the pigments (chlorophylls, pheophytins a and a', pyropheophytin a) first before HPLC. Reverse phase HPLC can be used to analyse the eluant, and the separated components are monitored at 410 nm using a photometric detector or fluorescence detector (excitation wavelength, λ_{ex}=430 nm and emission wavelength, λ_{em}=670 nm) (International Organization for Standardization., 2009a).

2.9.3 Carotenoids

β-carotene and lutein are the main carotenoids present in olive oil. There are some very small quantities of xanthophylls such as violaxanthin and neoxanthin. Total carotenoids may range between 1 and 20 mg/kg, but usually values do not exceed 10 mg/kg. Carotenoids are singlet oxygen quenchers and protect the oil from photo-oxidation. There is probably a relation between carotenoids and the mode of action of polar phenols and α-tocopherol (Boskou, 2011).

2.10 Deterioration of olive oil

The most important deterioration mechanisms of olive oil are hydrolysis and oxidation. Hydrolysis, also known as lipolysis, usually commences when the oil is still in the fruit, while oxidation proceeds after the oil is removed from the fruit and mainly during storage. Oxidation may occur either in the dark (autoxidation) or in the light (photooxidation) in the presence of air (Kiritsakis, 1998).

2.10.1 Hydrolysis

Hydrolysis causes release of fatty acids from the triacylglycerol molecule with consequent increase in total acidity (free fatty acids) and with change in flavour. Figure 7 shows a triacylglycerol (triglyceride) molecule hydrolysing into glycerol and free fatty acids (FFA), where R_1, R_2 and R_3 are fatty acid residues.

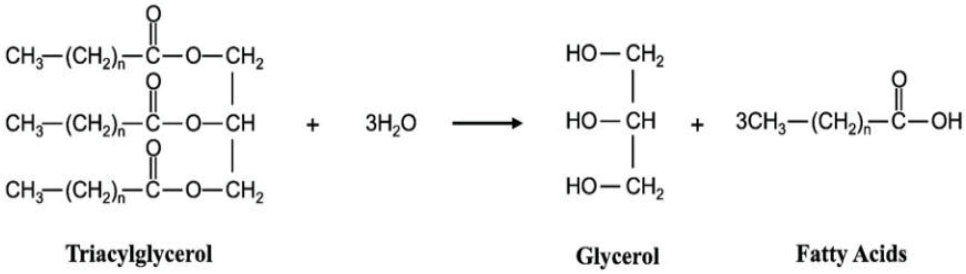


Figure 7. Olive oil lipolysis results from microbial and enzymic lipolysis (Lampidonis, et al., 2011).

2.10.1.1 Fatty acids

Chemically a fatty acid is a carboxylic acid. It is often with a carboxyl group at one end with a long unbranched aliphatic tail (chain), which is either saturated or unsaturated (Wiesman, 2009). In olive oil, most of the fatty acids are present as triacylglycerols, but a TAG unit may lose one fatty acid to become a diacylglycerol (DAG), or two fatty acids to become a monoacylglycerol (MAG) (Wiesman, 2009). The presence of free fatty acids in olive oil indicates that degradation through poor handling during processing and it is considered a sign of deterioration of oil quality (Wiesman, 2009).

The fatty acids found in olive oil include: myristic acid (C14:0); palmitoleic acid (C16:1); heptadecanoic acid (C17:0); heptadecanoic acid (C17:1); stearic acid (C18:0); oleic acid (C18:1); linoleic acid (C18:2); linolenic acid (C18:3); arachidic acid (C20:0); gadoleic acid (eicosenoic) (C20:1); behenic acid (C22:0); lignoceric acid (C24:0) (Fedeli, 1977). Table 6 shows the accepted limits for fatty acid composition of olive oil (Boskou, 1996a).

Table 6. Fatty Acid Composition of Olive Oil (Boskou, 1996a).

Fatty acid	Limits
Myristic (C 14:0)	0.0-0.1
Palmitic (C 16:0)	7.5-20.0
Palmitoleic (C 16:1)	0.3-3.5
Heptadecanoic (C 17:0)	0.0-0.5
Heptadecenoic (C 17:1)	0.0-0.6
Stearic (C 18:0)	0.5-5.0
Oleic (C 18:1)	55.0-83.0
Linoleic (C 18:2)	3.5-21.0
Linolenic (C 18:3)	0.0-1.5
Arachidic (C 20:0)	0.0-0.8
Eicosenoic (C 20:1)	Nonspecified
Behenic (C 22:0)	0.0-0.2
Lignoceric (C 24:0)	0.0-1.0

2.10.1.2 Glycerides

Glycerol can be esterified with one, two, or three fatty acids, and the individual fatty acids can be located on different carbons of glycerol (O'Keefe, 1998). In the numbering that describes the hydroxyl groups on the glycerol molecule in Fisher projection, *sn*1, *sn*2 and *sn*3 designations are used for the top (C1), middle (C2), and bottom (C3) OH groups (Figure 8). The *sn* term indicates stereospecific numbering.

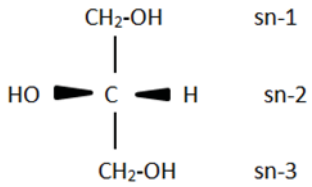


Figure 8. Stereospecific numbering (sn) of triacylglycerols (O'Keefe, 1998).

The glycerol unit can combine with any three of several fatty acids to form a triacylglycerol (TAG). The carbon chain of the fatty acids attached may be of different lengths, and they may be saturated, monounsaturated or polyunsaturated (Wiesman, 2009). The major triacylglycerols of the olive oils are oleic-oleic-oleic, palmitic-oleic-oleic, oleic-oleic-linoleic, and palmitic-oleic-linoleic (Fedeli, 1977; Wiesman, 2009). Biosynthesis of the TAG in olives generally follows 1,3 random distribution, which means that fatty acids on the TAG are randomly distributed in the 1 and 3 position on the molecule; in the 2 position there is always an unsaturated fatty acid (Wiesman, 2009).

Triacylglycerols (TAGs) account for around 98% of VOO lipid composition and can be defined as the VOO glycerolipid fraction together with diacylglycerols (DAGs) and monoacylglycerols (MAGs). The fatty acid (FA) composition and the different combination of FAs to form TAG molecules influence physico-chemical characteristics of a fat as well as its physiological properties (Vichi et al., 2012). For good-quality oil, three fatty acids should be bound and remain as part of the TAG (Wiesman, 2009) (Figure 9).

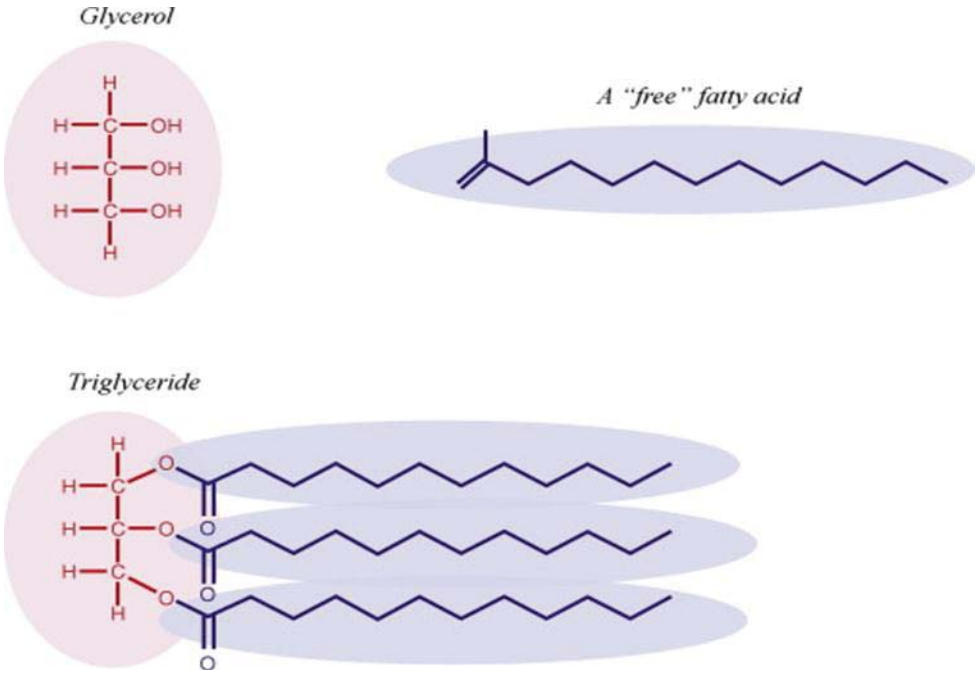


Figure 9. Structure of a molecule of glycerol, a free fatty acid, and a triglyceride (triacylglycerol) compound of olive oil (Wiesman, 2009).

2.10.1.3 Diacylglycerols

Diacylglycerols (or "diglycerides") are esters of the trihydric alcohol glycerol in which two of the hydroxyl groups are esterified with long-chain fatty acids. They can exist in three stereochemical forms (Figure 10).

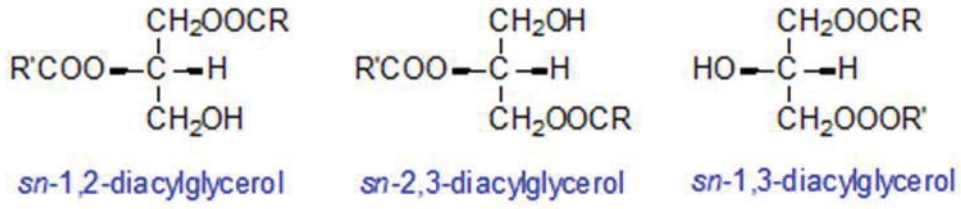


Figure 10. Three stereochemical forms of diacylglycerols (Lipid library, 2013) (Source:http://lipidlibrary.aocs.org/lipids/dg/index.htm)

The enzymatic glycerolysis and alcoholysis of triacylglycerols (TAG) to synthesize DAGs typically result in a mixture of mono-, di-, and tri-acylglerols with the DAG consisting of the regiospecific isomers 1(3),2-DAG (which will be referred to

throughout as 1,2-Diacylglycerols (1,2-DAG) and 1,3-Diacylglycerols (1,3-DAG). The synthesis is further complicated by the fact that the regiospecific sn-2 fatty acid of the 1,2-DAG isomer undergoes a spontaneous acyl migration from the sn-2 position to the sn-3 position to form the 1,3-DAG isomer (Kodali et al., 1990). It has been demonstrated that acid impurities, high polarity solvents, and higher temperatures increase the rate of the intramolecular isomerization from 1,2-DAG to 1,3-DAG (Kodali et al., 1990; Fureby et al., 1996).

The distribution of fatty acids varies according to the storage conditions of the olive oil. 1,2-DAG tend to isomerize to the more stable 1,3-DAG over time, which can give some information about the age of oil and storage conditions (Boskou et al., 2006a; Gunstone, 2004). The ratio of 1,2-/1,3-DAG is regarded as a useful criterion to monitor quality (Boskou et al., 2006a). The 1,3-/1,2-DG ratio is a maker for assessing the genuineness of virgin olive oils of low acidities, determining the oil aging, and evaluating the storage conditions if the DAGs determination is performed in the early stages after obtaining the oil (Pérez-Camino et al., 2001; Boskou et al., 2006a).

During the breakdown of triacylglycerols, diacylglycerols are formed. Fresh extra virgin olive oil contains a high proportion of 1,2-DAG. While olive oil from poor quality fruits and refined olive oils, they have elevated levels of 1,3-DAG. The ratio of 1,2-DAG to 1,3-DAG is an indicator for olive oil that is hydrolyzed, oxidized, of poor quality, and/or adulterated with refined oil (Frankel et al., 2010). As only 1,2-DAG is practically present in fresh oils whereas 1,3-DAG is formed during oil preservation, a high value of the ratio (1,2-/1,3-DAG) is related to very high quality olive oil (Angerosa et al., 2006). It has been shown that in virgin olive oil, DAGs are present between 1 to 3% (Kodali et al., 1990).

As 1,2-diacylglycerols will be transformed to the more stable 1,3-diacylglycerols during storage or due to acidic catalyzed reaction, the mass fraction of

1,2-diacylglycerols can be used as a quality criterion for vegetable fats and oils. According to international standard ISO 29822 (International Organization for Standardization., 2009b), the mass fraction of 1,2-diacylglycerols can be detected by gas chromatography. The degree of isomerisation is defined as the percentage of the peak areas of all 1,2-diacylglycerols relative to the sum of the peaks of all diacylglycerols. Miniaturized column chromatography on a silica gel column is used to separate the isomeric diacylglycerols as the more polar fraction from the major part of other lipids. The peak areas of 1,2- and 1,3-isomers are determined after silylation and then by gas chromatography (International Organization for Standardization., 2009b).

An elevated level of 1,2-diacylglycerol and pyropheophytins (PPPs) indicates the samples were oxidized, of poor quality, and/or adulterated with cheaper refined oils (Frankel et al., 2010). Standards for 1,2-diacylglycerol (DAGs) content and pyropheophytins (PPP) were developed by the German Fat and Oil Society (DGF) and were recently adopted by the German government and the Australian Olive Association (AOA) as useful tools to assess olive oil quality (Frankel et al., 2010).

2.10.2 Oil Oxidation

Oxidative stability is one of the basic parameters for assessing the quality of virgin olive oil mainly because it provides a reliable assessment of the vulnerability of the oil to oxidation changes, which are the main cause of its deterioration (Žanetić et al., 2013). While the process of oxidation for olive oil requires a few factors, one of the main causes of oil deterioration is the presence of oxygen (Morales & Przybylski, 2000). When atmospheric oxygen enters the lipid molecular moiety, lipid hydroperoxides can be formed, which lead to the ROOH (a hydroperoxide) compounds formed. There are three different mechanisms of oxidation giving rise to hydroperoxides: autoxidation, photo-oxidation and lipoxygenase oxidation (Bianchi,

2002). The most common oxidative process is autoxidation that occurs after an induction period (Hudson & Gordon, 1994).

2.10.2.1 Induction period

The course of the oxidation shows two distinct phases. During the first phase, the oxidation goes slowly and at a uniform rate (Hamilton, 1994). After the oxidation has proceeded to a certain point, it enters a second phase, which has a rapidly accelerating rate of oxidation, and the eventual rate is many times greater than that observed in the initial phase (Hamilton, 1994; Velasco & Dobarganes, 2002). The flavour of the oil starts to change at the beginning of the second phase, but some experts can taste the subtle changes in oil quality before this second phase is reached. The initial phase is called the induction period (Hamilton, 1994). During the induction period, the deterioration is not severe (Hudson & Gordon, 1994).

2.10.2.2 Autoxidation

The classical autoxidation route means lipid molecules RH produce free radicals R• interacting with oxygen in the presence of a catalyst, e.g. the initiation can be promoted by the action of external energy sources such as heat, light or high energy radiation or some other sources (Kiritsakis, 1998). The free radical R• produced in the initiation steps can then react to form a lipid peroxy radical ROO• which can react further to give the hydroperoxide ROOH (Kiritsakis, 1998). A further free radical R• can also be produced in the second reaction of the propagation steps, making it a self-propagating chain process. A small amount of catalyst, e.g. copper ions can initiate the second reaction occurring, which can produce many hydroperoxide molecules that ultimately break down to cause rancidity (Gunstone, 2008). In the termination reactions, when two radicals combine to give products, the self-propagating chain can be stopped (Hamilton, 1994; Gunstone, 2008). The three steps initiation, propagation and termination are shown in Figure 11.

Initiation: $RH+O_2 \xrightarrow{catalyst} R\bullet+\bullet OOH$

$RH \xrightarrow{catalyst} R\bullet+\bullet H$

Propagation: $R\bullet+O_2 \longrightarrow RO_2^{\bullet}$

$RO_2^{\bullet}+RH \longrightarrow RO_2H+R\bullet$

Termination: $R\bullet+ R\bullet \longrightarrow R\text{—} R$

$RO_2^{\bullet}+ R\bullet \longrightarrow RO_2R$

RH= unsaturated lipid, R•=lipid radical and $RO_2^{\bullet}$=lipid peroxy radical.

Figure 11. Autoxidation Mechanism (Boskou, 1996c; Kiritsakis, 1998; Gunstone, 2004).

Autoxidation is a radical chain process, which means the intermediates are radicals (odd electron species). Most importantly, the process will be inhibited by having fewer initiation steps or more termination steps resulting in fewer and shorter propagation cycles (Gunstone, 2008).

2.10.2.3 Photo-oxidation route

Different hydroperoxides are formed when light and certain photosensitiser molecules are present, photo-oxidation has been recognised as an alternative to the free radical mechanism (Hamilton, 1994). Photo-oxidation is a quicker reaction between olefin and a light-activated form of oxygen. The activation process requires a sensitiser such as chlorophyll, riboflavin, myoglobin, erythrosine, rose bengal, or methylene blue (Bianchi, 2002; Gunstone, 2008). These sensitisers can absorb energy from a photon and this energy eventually is passed to oxygen. These sensitisers raise the oxygen of triplet state to the more reactive singlet state. Singlet oxygen reacts rapidly with double bonds by an “ene” reaction to give an allylic hydroperoxide (Bianchi, 2002). Photo-oxidation is different from autoxidation, as photo-oxidation is faster and its rate is not related to the number of doubly allylic functions but the number of double

bonds (Gunstone, 2008). Photo-oxidation can be inhibited by appropriate quencher molecules such as carotene rather than by the range of compounds that inhibit autoxidation (Gunstone, 2008).

Extra virgin olive oil is subject to photo-degradation, resulting in a change in colour, followed by the development of undesirable odour and flavour (Kiritsakis, 1998). Light causes significant deterioration of olive oil quality in the presence of oxygen. Under the action of light, the four pigments (chlorophyll a and b, and pheophytin a and b) present in olive oil develop an oxidizing effect, while in the dark they act as antioxidants, acting synergistically with phenolic antioxidants (Kiritsakis, 1998).

Chlorophyll pigments are responsible for the greenish hues in virgin olive oil. Their content may range from 10 to 30 mg/kg. The main chlorophyll in packed oil is pheophytin a (Boskou, 2011). Chlorophylls may act as weak antioxidants when the oil is stored in the dark. However, light acts as strong oxidation promoters. Chlorophylls and pheophytins promote photo-oxidation of refined oils (Boskou, 2011). They act as singlet oxygen quenchers in natural olive oil, a pronounced effect of the action of singlet oxygen may be observed in natural olive oils (Boskou, 2011).

2.10.2.4 Lipoxygenase route

The lipoxygenase oxidation reaction is common with many enzyme reactions, but the basic stoichiometry of it is the same as for autoxidation (Hamilton, 1994; Gunstone, 2008). Primarily, the oxidation is concerned with the unsaturated fatty acids, of which oleic (V), linoleic (VI) and linolenic (VII) acids are the most abundant (Hamilton, 1994). Lipoxygenase is very specific about the substrate and how the substrate is oxidised. Linoleic acid is oxidised at positions 9 and 13 by lipoxygenase isolated from most natural sources (Bianchi, 2002; Gunstone, 2008).

2.10.2.5 Decomposition products

There are three recognized stages during oxidation and tests are available for each stage (Gunstone, 2008):

1. Primary products of oxidation are allylic hydroperoxides and are measured as peroxide values or as conjugated dienes formed during oxidation of polyunsaturated fatty acids (PUFA).
2. Secondary products are mainly unsaturated aldehydes and are measured by the anisidine value.
3. Tertiary oxidation products include short-chain acids measured by the Rancimat or OSI (oil stability index) or malondialdehyde measured by the TBA (thiobarbituric acid) test.

2.10.2.6 Peroxide and anisidine value

The common method of assessing oxidative status is by measuring hydroperoxides which can react with acidified potassium iodide to liberate iodine. Free iodines can be measured volumetrically by reaction with sodium thiosulphate (Kiritsakis, 1998). Refining destroys hydroperoxides which will be cleaved to aldehydes, but it does not regenerate the fat in its original form (Akoh & Min, 1997).

Peroxide values indicate formation of primary products of oxidative spoilage (peroxides and hydroperoxides) (Žanetić, et al., 2013). Quantitation of hydroperoxides is the classical method of the determination of peroxide value (PV) (Akoh & Min, 1997). The hydroperoxide content is determined by an iodometric method which is based on the reduction of the hydroperoxide group (ROOH) with iodide ion (I^-). The amount of iodine (I_2) liberated is proportional to the concentration of peroxide present. Released I_2 is assessed by titration against a standardized solution of sodium thiosulfate ($Na_2S_2O_3$) by a starch indicator (Akoh & Min, 1997). Chemical reactions involved in PV determination are given as follows:

$2ROOH+2H^{+}+2KI \rightarrow I_2 + 2ROH+ H_2O + K_2O$

$I_2 + 2Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2NaI$

(Akoh & Min, 1997).

While volatile aldehydes are removed during subsequent refining, short-chain aldehydes attached to the glycerol moiety remain (sometimes called core aldehydes) and can be detected by the anisidine value (Gunstone, 2008). Refining oxidised oils will reduce the peroxide value but the anisidine value will not be reduced to zero (Gunstone, 2008). These two measurements peroxide and anisidine values may be combined in a total oxidation value representing the sum of twice the peroxide value plus the anisidine value (Gunstone, 2008).

2.11 Phenolic Compounds

Virgin olive oil is known be more resistant to oxidation than other edible oils because of their content of natural antioxidants and lower unsaturation levels (Cinquanta, et al., 2001). The stability of virgin olive oils is due to their natural phenolic compounds, since these compounds are able to donate a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation (Morales & Przybylski, 2000; Cinquanta, et al., 2001). Phenolic compounds are transferred into the oil during the olive processing, but their concentration is dramatically reduced during refining and storage of oils (Gutiérrez et al., 2001; Angerosa, et al., 2006).

2.12 Bitterness

The bitterness taste of virgin olive oil, if not excessive, is a positive attribute and is related to the concentration of phenolic compounds (Angerosa et al., 2001; Boskou et al., 2006b; Kalua et al., 2007). Phenolics are mainly responsible for the shelf-life of the oils and also for their typical bitter taste (Angerosa et al., 2001; Boskou et al.,

2006b). Gutierrez-Rosales et al. (2003) have correlated bitter intensity of many virgin olive oil samples with the level of individual phenols (Boskou et al., 2006b). According to Boskou et al. (2006b), the dialdehydic and aldehydic forms of decarboxymethyl-oleuropein aglycone and the dialdehydic form of decarboxymethyl-ligstroside aglycone are responsible for the bitterness.

2.13 Avocado oil

Avocado oil has a very similar fatty acid profile to olive oil (Woolf et al., 2009). The international quality standards for extra virgin avocado oil have been recommended (Woolf et al., 2009). The standards recommended for extra virgin avocado oil are free fatty acid (% as oleic acid) $\leq$ 0.5%, peroxide value $\leq$ 4 meqO_2/kg (Woolf et al., 2009). The avocado oil contains considerably high levels of oleic acid (~ 60%) and natural antioxidants such as tocopherols and phenolic compounds (Rodríguez-Carpena et al., 2012). Avocado oil also contains high levels of chlorophyll and carotenoid pigments (Woolf et al., 2009; Knothe, 2013).

2.14 Conclusions from literature review

Extra virgin olive oil is the highest quality olive oil grade.

Only Australia's quality criteria standards have the quality standards of pyropheophytin a and 1,2-diacylglycerols for extra virgin olive oil compared with the IOC and USA standards.

Pyropheophytin a is the thermal degraded product of chlorophyll, which can be detected. The ratio between pheophytin a and pyropheophytin a is an indicator of storage abuse and adulteration.

1,2-diacylglycerols tend to isomerize to the more stable 1,3-diacylglycerols during olive oil storage. The ratio between them determines the oil aging and the genuineness of virgin olive oil.

Oxidative reactions occur in olive oil during storage and can be monitored with peroxide value. Peroxide value, total phenolics and bitterness can be tracked during olive oil storage and they can be the chemical parameters of olive oil quality.

Chapter Three: Materials and Methods

3.1 Storage trial

3.1.1 Olive oil

Olives were harvested during April/May 2012 and processed immediately. The oils were stored under nitrogen then bottled for use in the shelf life trials from 8th November 2012. Olive oil was extracted at Matiatia Grove olive press on Waiheke using a Perilisi malaxer and decanter system. Oils from two cultivars were supplied. They were 'Frantoio' and 'Leccino'. The oils were supplied in dark green bottles (nitrogen sparged) and stored at 4 ± 1°C until the storage trial was set up on 8 November, 2012.

3.1.2 Storage trial

The olive oils were decanted into 50 mL dark brown bottles, nitrogen sparged and sealed and stored at the following storage temperatures for the duration of the storage trials; 7 ± 1 °C, 20 ± 1 °C, 30 ± 1 °C and 40 ± 1 °C. All bottles were stored together for each temperature. At each sample day, one oil sample was removed for each test. The oils were stored in incubators (Qualtex, Andrew Thom Limited, Sydney, Australia, Model: 65R2) set at the required temperatures. At each sampling day the oils from the 50 mL bottles was decanted into two 25 mL dark brown bottles (approximately 20 mL oil) and one 5 mL brown bottle (approximately 5 mL). The oils from the two 25 mL dark brown bottles were used for peroxide value, %free fatty acid, bitterness, total phenolics and K value test while the oils from the 5 mL brown bottle were used for the diacylglycerols (DAGs) test. The oil left in the 50 mL bottles (approximately 5 mL) was used for pyropheophytins (PPPs) test on the sampling day. The oils which were not analysed on the sampling day were stored at -80 ± 1 °C until they were analysed. The oils were analysed for the following tests: pyropheophytins

(PPPs), peroxide value, %free fatty acid, bitterness, total phenolics, K value and the percentage of diacylglycerols (DAGs).

The timeline is shown in Table 7. The "√" indicates when the oils were removed from the incubator, sampled and tested.

Table 7. The timeline for the storage oil tests.

Time (days)	7 °C	20 °C	30 °C	40 °C
0				
7			√	√
14		√	√	√
21			√	√
28	√	√	√	√
42		√	√	√
56	√	√	√	√
70		√	√	√
84	√	√	√	√
98		√	√	√
112	√	√	√	√
140	√	√	√	
168	√	√	√	

√ - indicates when oils were sampled and tested.

3.1.3 Commercial oil samples.

Commercial olive oil samples were obtained from the 2012 Olives NZ judging event. Selected oils were chosen at random to represent various cultivars and regions of New Zealand. Other oils were purchased from Countdown supermarkets in Auckland. Details of all commercial oils are given in the Table 8 which provides the information of awards EVOOs and the commercial oils tested.

Table 8. Commercial olive oil information.

a. Oils submitted to Olives Awards

Number	Regions	Pressing date	Best Before date
NZ 'Frantoio' 1	Auckland, New Zealand	Jul-12	No date
NZ 'Leccino' 1	Wairarapa, New Zealand	Jun-12	Best before June 2014
NZ Blend 1	Northland, New Zealand	Harvest 05/12	No date
NZ Blend 2	Bay of Plenty, New Zealand	09/05/12	Best before 30-04-2014
NZ Blend 3	Kapiti Coast New Zealand	No date	No date
NZ 'Frantoio' 2	Northland, New Zealand	May-12	Best before May 2014
NZ 'Leccino' 2	Hawkes Bay, New Zealand	Jul-12	Best before July 2014
NZ 'Leccino' 3	Bay of Plenty, New Zealand	Jul-05	Best before 30-04-2014

b. Supermarket bought olive oils

Brand	EVOO	Source of oil	Best before date
Australia 1	yes	Australia	3-Oct-14
Italy 1	yes	Italy	20-Feb-14
Italy 2	yes	Italy	Oct-14
Italy 3	yes	Italy	18-Mar-14
Spanish 1	yes	Spain	28-Sep-13
NZ 'Frantoio' 3	yes	New Zealand	30-Jun-14
NZ Blend 4	yes	New Zealand	Jul-14
Avocado oil			
Avocado oil 1	EV	New Zealand	6/02/2018
Avocado oil 2	EV	New Zealand	7-Feb-15

3.2 Analytical methods

3.2.1 Peroxide Value

3.2.1.1 Reagents

Potassium iodide: Analytical Reagent Grade, Lab Serv ^{TM}, Thermo Fisher Scientific, New Zealand.

Isooctane (2,2,4-trimethyl pentane): HPLC grade, Fisher Scientific, UK.

Hydrochloric acid: LabServ Analytical Grade, ThermoFisher Scientific, New Zealand.

Glacial acetic acid: Analytical reagent grade, Fisher Scientific, UK.

Sodium carbonate anhydrous: Analytical Reagent UNIVAR Ajax Finechem Pty Ltd, New Zealand.

Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$): Scharlau reagent grade, ACS, European Union.

Potassium iodate (KIO_3): Analytical Reagent Grade, LabServ^{TM}, Thermo Fisher Scientific, New Zealand.

Starch: AnalaR grade reagent, BDH, England.

3.2.1.2 Preparation of reagents

Fresh saturated potassium iodide (KI) solution: 10 g of the potassium iodide was dissolved into 5 mL distilled water, stirred thoroughly and then ultrasonicated for a further 5 minutes. There were undissolved crystals after stirring and ultrasonication, which was an indication of a saturated solution. The fresh saturated potassium iodide (KI) solution was made daily.

Starch indicator: 1 % (w/v) solution of starch was prepared by adding 1 g unmodified starch to 100 mL distilled water and the solution was boiled for a further two minutes. This solution was stored at 4 - 10 °C no longer than three weeks.

Glacial acetic acid and isooctane solution: the solution was made up in a 3:2 volumetric ratio. Dried potassium iodate (KIO_3): potassium iodate was dried for two hours at 110 °C and cooled in a desiccator.

3.2.1.3 Standardisation of sodium thiosulfate (0.002 N)

Firstly, making 0.1 N sodium thiosulfate solution: 24.817 g $Na_2S_2O_3 \cdot 5H_2O$ and 0.05 g sodium carbonate were dissolved in 1 L freshly boiled and cooled distilled water. This solution was diluted 50 times with cooled distilled water and made up to 500 mL. The standardisation procedure for 0.002 N $Na_2S_2O_3 \cdot 5H_2O$ solution was as follows:

accurately weighed (0.002 – 0.003 g) dried KIO_3, which was dissolved in 75 mL distilled water in a 250 mL conical flask and then 0.04-0.06 grams iodate free KI solution was added. After an aliquot of 2 mL 6 M HCL was added, the solution was immediately titrated using sodium thiosulfate solution with gentle shaking until the colour of the solution turned pale yellow. A solution containing 1 % starch (1 mL) was added and the solution was titrated until the violet colour disappeared. The final volume of sodium thiosulfate was recorded and a blank titration without KIO_3 was conducted. Titrations were carried out in triplicate. The normality (N) of sodium thiosulfate was calculated.

$$\text{Normality, N} = \frac{28.03 \times \text{W}}{\text{S} - \text{B}}$$

Where W is the weight of KIO_3 in grams, *S* and *B* are the volume of sodium thiosulfate required to titrate the sample and the blank, respectively.

3.2.1.4 Procedure for determination of Peroxide Value

Peroxide value (PV) was determined by the AOCS Official Method Cd 8b-90, acetic acid-isooctane method (AOCS, 1998a). Olive oil was accurately weighed (2 ± 0.5 g) into a 250 mL stoppered Erlenmeyer flask. The acetic acid-isooctane solution (30 mL) was then added to facilitate dissolving of oil with swirling. Saturated potassium iodide solution (0.5 mL) was added to the flask in the dark. After exactly 1 minute, 30 mL distilled water was then added. The indicator of 1 % starch (1 mL) was added to this solution before titration. The solution was titrated with 0.002 N sodium thiosulfate with constant shaking of the solution to the end point to liberate all iodine from the acetic acid-isooctane layer. Sodium thiosulfate solution was added drop wise until the violet colour disappeared. Titrations were carried out in duplicate and a blank titration was conducted.

3.2.1.5 Expression of result

The peroxide value (PV) was calculated as

$$\mathrm{PV} = \frac{(\mathrm{S} - \mathrm{B}) \times \mathrm{N} \times 1000}{\text{Weight of oil, g}}$$

Where S and B are titration volumes (mL) of the sample and blank respectively, and N is the calculated normality of sodium thiosulfate solution. The result was reported and expressed as the milliequivalents of active oxygen per kg olive oil ($meqO_2/kg$) ± SEM

3.2.2 Analysis of free fatty acids (FFA)

3.2.2.1 Reagents

Phenolphthalein indicator: Acros Organics, USA.

Ethanol: LabServ Analytical, Reagent Grade, New Zealand.

Sodium hydroxide (NaOH): Univar, Analytical reagent, New Zealand.

Potassium hydrogen phthalate: Analytical grade ,LabServeTM, New Zealand.

3.2.2.2 Preparation of reagents

Phenolphthalein indicator (1%): 1 g of phenolphthalein was dissolved in 100 mL 98% ethanol.

Carbon dioxide free distilled water: boiling distilled water for 2 minutes.

Sodium hydroxide solution (0.05 M): 2 g of sodium hydroxide pellets dissolved into 1 L of carbon dioxide free distilled water and followed by standardization with the AOCS official method Ca 5a-40 (AOCS, 1998b).

Sodium hydroxide solution (0.01 M): diluting the 0.05M Sodium hydroxide solution for 5 times by cooled distilled water.

Potassium hydrogen phthalate: dried in an oven at 120 ± 0.5 °C for two hours and cooled in a desiccator.

Standardisation of sodium hydroxide (0.01 M)

Dried potassium hydrogen phthalate (0.05 ± 0.005 g) was accurately weighed in an Erlenmeyer flask and then dissolved with distilled water (25 mL). Three drops of phenolphthalein indicator was added to this solution. The solution was standardized by titration with the NaOH solution until the first persistent faint pink colour was obtained. The initial and final titrated volume (mL) of NaOH was recorded. Titrations were completed in triplicate. The molarity of NaOH (mol/L) was calculated.

$$\text{Molarity}\left(\frac{\text{mol}}{\text{L}}\right) = \frac{\text{Weight, g of potassium pthalate} \times 1\ \text{mol} \times 1000\ \text{mL}}{\text{mL NaOH} \times 204.229\ \text{g} \times 1\ \text{L}}$$

Where, 204.229 g/mol is the molecular mass of potassium phthalate. The standardised NaOH solution (0.05 ± 0.01M) was used as the titrant in determination of FFA.

3.2.2.3 Procedure for the determination of FFA

The percentage of free fatty acids in the oil was determined following the AOCS official method Ca 5a-40 (AOCS, 1998b).

Neutralised 98 % ethanol: three drops of phenolphthalein indicator was added to 50 mL 98 % ethanol in a conical flask and then immediately titrated with standardised NaOH solution (0.01 M) until the ethanol produced a faint but permanent pink colour.

A sample of olive oil (2 ± 0.05 g) was accurately weighed into a 250 mL conical flask and then the above neutralised ethanol was added and followed by addition of 1-2 drops of phenolphthalein indicator. The solution was heated to boiling and then immediately titrated with gentle shaking using the standardised 0.01 M NaOH until a

persistent faint pink colour remained in the alcohol layer for 30 seconds. Titrations were carried out in triplicate.

Expression of the results

The percentage of free fatty acids was calculated by the formula

$$\text{FFA } \% = \frac{\text{Titre (mL)0.05 M NaOH} \times \text{molarity}* \text{ of NaOH} \times \text{molecular weight of oleic acid (282)}}{\text{Weight of olive oil sample,g} \times 10}$$

* The molarity of standardised NaOH. The result was reported as mean FFA (expressed as % oleic acid) ± standard error.

3.2.3 Specific extinction coefficient, K values

The spectrophotometric examination was carried out following the International Olive Council methodology (COI/T20/Doc.no. 19/Rev.3) (International Olive Council, 2010b).

3.2.3.1 Reagents

Isooctane (2,2,4-trimethylpentane): HPLC grade, Fisher Scientific, UK.

3.2.3.2 Procedure

An olive oil sample was accurately weighed (0.25 ± 0.05 g) into a 25 mL volumetric flask and then made up to the mark with the isooctane solvent. The solution was gently swirled to ensure homogeneity. The sample solution was then poured into a standard rectangular quartz cuvette cell (I-Q-100 mm, Scientific Supplies) having an optimal length of 1 cm with the solution obtained. The specific extinction coefficient of the clear solution was recorded at the wavelengths 228, 232, 236, 264, 266, 268, 270, 272 and 274 nm respectively (Shimadzu UV-Visible spectrometer, UV-1601, Japan). Absorbance of the pure isooctane solvent was used as the reference.

3.2.3.3 Expression of the results

The specific extinctions from 228 nm to 274 nm were calculated using the formula

$$K = \frac{A}{bc}$$

where K is the specific extinction, A is the specific absorbance measured at wavelength 270 nm and 232 nm, b thickness of the cuvette in cm and c is concentration of the solution in g/100 mL (c=1 when 0.25 g/25 mL).

The extinction value at K_{232} nm was corrected for esters using 0.07 as the correction factor (Kiritsakis, 1998). By this correction factor, the percentage of conjugated dienes was expressed as

$$(K_{232} - 0.07) \times 0.91$$

Kiritsakis (1998) reported this correction factor to provide an indication of the degree of oxidation of olive oil. All results are reported as mean values ± SEM.

3.2.4 Determination of the degradation products of pheophytins a, a' and pyropheophytins

3.2.4.1 Reagents

Standard Chlorophyll a: ChromaDex, USA.

Acetone: Fisher Scientific, Optima®United States.

Methanol: Fisher Scientific, Optima®, Trinidad.

Diethyl ether: Lab Serv™, Analytical Reagent Grade, New Zealand.

Petroleum ether (boiling point range 40 °C to 60 °C): Fisher Scientific, Analytical reagent grade, UK.

Heptane: HPLC grade, Prolabo,VWR™, EC.

Silica gel 60 (0.063-0.100 mm): Merck Chemicals for Column Chromatography.

3.2.4.2 Preparation of reagents

Petroleum ether and diethyl ether solvent mixture: Petroleum ether was mixed with diethyl ether solvent in the volumetric ratio 9:1.

Silica gel 60: silica gel was dried at 50 °C and then 5% hydrated with distilled water.

Mobile phase: distilled water was mixed with methanol and acetone in the volumetric ratio 1: 9:15. The mobile phase was filtered through 0.22 μm nylon filters (Millipore Durapore membrane filter, 0.22 μm GV) and degassed before to use.

Silica gel column: a small stopper of cotton wool approximately 5 mm high was inserted into the lower part of an empty 5 mL pipette tip followed by 1 g silica gel 60 (5% hydrated). On top of the silica gel was placed another 5 mm high stopper of cotton wool. The filling was compressed slightly by pressing down on the cotton wool with the flat end of a stirring rod.

3.2.4.3 The HPLC chromatographic condition (Shimadzu Model SCL-10A) HPLC System

The Shimadzu HPLC system (Shimadzu Model SCL-10A, Japan) was used for separation of the pigments (chlorophylls, pheophytins a and a', pyropheophytin a). The system was equipped with a model SCL-10AVP system controller, a model LC-10A pump, an auto injector SIL-10AF, a model SPD-M10AVP detector for measurements at a wavelength of 410 nm. The size of the HPLC column used was 150×4.60 mm, 5 micron filling with Gemini reverse-phase type C18, particle size 5 μm, pore size 110A. The volume of sample injected was 10 μL at a flow rate of 1.0 mL/min and the temperature of the column was 30 ± 1 °C. The UV detector was set at 410 nm.

3.2.4.4 Separation of chlorophyll a, pheophytin a, pheophytin a' and pyropheophytin

Heating can lead to chlorophyll degradation due to loss of the magnesium ion, also the formation of pyropheophytin is the result of loss of the carbomethoxy group (Van Boekel, 2000). The standard chlorophyll a was run to determine its retention time. The extra virgin olive oil was treated by heating at different temperatures for chlorophyll degradation. The trial of heating temperatures was set at 80 °C (heating for 30 minutes and 60 minutes), 100 °C (heating for 60 minutes), 120 °C (heating for 30 minutes and 60 minutes) and 160 °C (heating for 30 minutes and 60 minutes) in order to identify the percentage changes of chlorophyll a, pheophytin a, pheophytin a' and pyropheophytin in total chlorophyll. In this way, the peak of chlorophyll a, pheophytin a, pheophytin a' and pyropheophytin could be identified.

3.2.4.5 Sample preparation procedure

An oil sample, 300 mg was weighed into a small beaker and dissolved in 1 mL of n-heptane. This solution was then poured onto the silica cartridge (silica gel column). The beaker was then rinsed twice with 1 mL portions of petroleum ether, pouring these washings on to the cartridge (column) also. As soon as the solvent had drained from the top of the cartridge (column), the non-polar substances were eluted twice with 5 mL petroleum ether/diethyl ether solvent mixture each time and the eluent was discarded. The pheophytin fraction on the silica was next eluted twice with 5 mL acetone and this fraction was collected in a pear-shaped flask protected from light. The obtained sample and solvent was evaporated to dryness on a rotary evaporator at a maximum of 20 °C. The residue was dissolved in 400 µL acetone. This solution was introduced immediately into the HPLC setup (the conditions were mentioned in section 3.2.4.3 HPLC conditions). No samples were held for more than 30 minutes before injection into the HPLC.

3.2.4.6 Analysis of results

The chlorophyll a, pheophytin a, pheophytin a' and pyropheophtin were identified by their retention time and peak areas. The peak areas were used to calculate the relative proportions of the analytes in the sample solution (International Standard, 2009a). The content as a percentage mass fraction of pyropheophytin a was calculated on the bases of a peak area ratio:

$$\omega_{PPPa} = \frac{A_{PPPa} \times 100}{(A_{PPPa} + \sum A_{PP})}$$

Where

A_{PPPa} is the peak area of pyropheophytin a;

ΣApp is the sum of the peak areas of chlorophyll a, pheophytin a and a'.

3.2.5 Determination of total phenolics

Total phenolic content was determined spectrophotometrically by the method of Folin-Ciocalteu (Singleton et al., 1998). Folin-Ciocalteu reagent is a complex mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidants (Singleton et al., 1998). The assay works by measuring the amount of test compounds needed to inhibit the oxidation of the Folin-Ciocalteau reagent (Vinson et al., 2005).

3.2.5.1 Reagents

Folin-Ciocalteu reagent: 2 M acid Bio-reagent grade, Sigma, USA.

Caffeic acid: Sigma Aldrich, Switzerland.

Methanol: Fisher Scientific, Optima®, Trinidad.

Formic acid: AnalaR®, BDH, England.

n-Hexane: Macron™ Chemicals, UltimAR®, USA.

Sodium carbonate : Analytical Reagent, Univar, New Zealand.

3.2.5.2 Preparation of reagents and caffeic acid standard

Acidified water (pH 2.5): formic acid was added into distilled water and then adjusted to pH 2.5 ± 0.5.

A mixture of methanol and acidified water solution: the ratio of methanol and acidified water was 80:20 (v/v).

A stock solution of Folin-Ciocalteu reagent: 0.2 M Folin-Ciocalteu reagent was prepared by diluting the 2 M Folin-Ciocalteu reagent by 10 times with distilled water.

Sodium carbonate solution (75 g/L): the sodium carbonate anhydrous was weighed and dissolved with distilled water.

Standard caffeic acid: caffeic acid was diluted in absolute methanol to prepare the concentration of 0.5 mg/mL. This standard stock solution was stable for several weeks at -20 °C.

3.2.5.3 Extraction of phenolic compounds in oil

5 g olive oil was weighed and the exact weight recorded. The sample was dissolved by using 10 mL of n-hexane to transfer it from beaker to separating funnel. To extract the phenolic compounds, the oil-hexane mixture was then added to 30 mL methanol-acidified water mixture by using a volumetric measuring cylinder and shaken vigorously for 3 minutes with gas releasing intermittently. The aqueous layer at the bottom of the separating funnel was then collected in a 250 mL round bottomed flask. This extraction cycle was repeated twice in order to extract almost all phenolics in the oil (Abramovic et al., 2007). The combined aqueous fraction was condensed in

a rotary evaporator (Büchi - RE 111 Rotavapor, Germany) under vacuum at 40 ± 1 °C. The dry residue was then diluted in 3 mL of absolute methanol and gently mixed thoroughly. This extract was used for the total phenolic assays following appropriate dilutions.

3.2.5.4 Procedure for total phenolic essay

Phenolic compounds in virgin olive oil are known to possess antioxidant properties which can delay the oxidation of the oil (Gambacorta et al., 2012). One of the most widely used methods for the routine determination of total phenolics in olive oil is the colorimetric assay based on the reaction of Folin-Ciocalteu (F-C) reagent with the functional hydroxyl groups of the analytes (Fuentes et al., 2012). A range of dilutions of the appropriately diluted extraction were prepared in 15 mL glass tubes: 100 µL, 125 µL, 200 µL of olive oil extraction were added respectively and absolute methanol was then added to make up to 1 mL volume. Folin-Ciocalteu reagent (5 mL) was added into each sample and sodium carbonate solution (4 mL) was then added within three to eight minutes after of addition of Folin-Ciocalteu reagent. The tubes were then sealed and incubated for 90 minutes in the dark at 20 °C.

A range of dilutions of standard caffeic acid solution (0.5 mg/mL) was prepared by pipetting the following volumes of the stock standard solution and making up to 1 mL with absolute methanol: 0.05 mL, 0.1 mL, 0.125 mL, 0.15 mL, 0.175 mL, 0.2 mL, 0.225 mL and 0.25 mL. Folin-Ciocalteu reagent (5 mL) was added into each of the tubes followed by the addition of 4 mL sodium carbonate solution in the same manner as mentioned previously. The capped tubes were incubated in the dark at 20 °C and absorbance at 760 nm was read after 90 minutes.

3.2.5.5 Calculations and presentation of results

The total phenolic content was calculated using the equation for the straight line standard curve obtained from plotting the absorbance values (at 760 nm) against concentration of caffeic acid.

$$\text{Absorbance (760 nm)} = \frac{\text{Slope (mg caffeic acid)}}{\text{mL MeOH}} + \text{ intercept}$$

Thus from the standard curve mg caffeic acid/mL MeOH was obtained and the total phenolic content was calculated as below

$$\frac{\text{mg caffeic acid}}{\text{mL MeOH}} \times \text{dilution factor} \times \frac{3\text{ mL MeOH}}{1} \times \frac{1}{\text{weight oil, g}} \times 1000 = \frac{\text{mg total phenolics}}{\text{kg oil}} \text{ (caffeic acid equivalent)}$$

The result was reported as milligram of total phenolics (caffeic acid equivalent) per kilogram of the oil, as caffiec acid is one simple phenolic found in virgin olive oil (Papadopoulos & Boskou, 1991).

3.2.6 Determination of the Bitter Index (K_{225})

3.2.6.1 Reagents

Isooctane (2,2,4-trimethylpentane): HPLC grade, Fisher Scientific, UK.

Methanol: Fisher Scientific, Optima®, Trinidad.

3.2.6.2 Procedure

Weigh accurately 0.25 g of the olive oil sample and use 5 mL n-hexane to dissolve. The extraction solvent MeOH/H_2O (60:40 v/v), 5 mL, was added into the oil-hexane mixture The mixture was vortexed for two minutes and centrifuged at 2470 × g for 10 minutes. After centrifuging, the hexane layer was removed, transferring the polar fraction into 25 mL volumetric flask. The extraction was repeated three times because preliminary results showed greater than 96% of polar phenolics were extracted by the

third extraction cycle. The combined polar fraction in a 25 mL volumetric flask was made up to volume with MeOH/H_2O (60:40, v/v). The UV-1601 spectrophotometer (Shimadzu Co., Kyoto, Japan) was used to detect the absorbance of this fraction with the wavelength of 225 nm. The 1cm quartz cuvette cell was filled with the combined polar fractions and the MeOH/H_2O (60:40, v/v) was the reference.

Calculation of bitter index (K_{225}):

K_{225} was calculated from the formula

$$K_{225}= \frac{As \times 1g}{Ws \times d}$$

Where As is absorbance of the polar extract; Ws is weight of oil in grams and d is the dilution factor corresponding to absorbance As, since the final result is expressed as 1% or absorbance of 1 g in 100 mL. The intensity of bitterness (IB) was then calculated from the expression reported by Gutiérrez-Rosales et al. (1992).

$$IB=13.33K_{225}-0.837$$

3.2.7 Analysis of 1,2- and 1,3-diacylglycerols

3.2.7.1 Reagents

Dilaurin Mixed Isomers ≥ 99%, sigma-aldrich, USA.

Dipalmitin ≥ 99.0%, sigma-aldrich, USA.

1,2-distearoyl-rac-glycerol ≈ 99%, sigma-aldrich, USA.

Dioleoylglycerol ≥ 99%, mixture of 1,3-and 1,2-isomers, sigma-aldrich, USA.

Diethyl ether: Lab Serv™, Analytical Reagent Grade, New Zealand.

Acetone: Fisher Scientific, Optima®United States.

Silica gel 60 (0.063-0.100 mm): Merck Chemicals for Column Chromatography.

Isooctane (2,2,4-trimethylpentane): HPLC grade, Fisher Scientific, UK.

Di-isopropyl ether: Analytical reagent grade, FisherScientific, UK.

Solvent mixture: the ratio of isooctane and diisopropyl ether was 85:15 (v/v).

Toluene: Multisolvent HPLC grade, Scharlau, Spain.

Silylating reagent: HMDS+TMCS+Pyridine, SUPELCO, USA.

3.2.7.2 Procedure

a. Preparation of the silica gel chromatography column

A small stopper of cotton wool approximately 5 mm high was inserted into the lower part of an empty 5 mL pipette tip followed by 1 g silica gel 60 (previously dried and hydrated as described in Section 3.2.4.2). On top of the silica gel was placed another 5 mm high stopper of cotton wool. The filling was compressed slightly by pressing down on the cotton wool with the flat end of a stirring rod.

b. Separation of the fraction containing non polar lipids

A 0.1 g oil sample was weighed into a beaker and 1 mL toluene was added. The test portion was transferred to the silica column followed by 1 mL solvent mixture (Section 3.2.7.1). The column was washed with two 3.5 mL portions of the solvent mixture. The pipette tip was washed with solvent mixture and the solvent discarded. The diacylglycerols was eluted with 2.5 mL portions of diethyl ether and the eluate was collected in a test tube. The solvent was removed from the eluate until it was dry with a stream of oxygen free nitrogen from a nitrogen cylinder. After this step, 200 μL silylation reagent was added to the test tube. The test tube was sealed and stood for 20 minutes at room temperature. After silylation, 1 mL acetone was added. The test tube was left to stand for 10 minutes until the precipitate formed. The top clear solution was transferred to a GC vial before injection into the gas chromatograph.

3.2.7.5 The conditions of gas chromatography

Function	Condition
Capillary GC column	Restek RTX5, 60 m; internal diameter 0.25 mm, film thickness 0.1 μm
Injection volume	1 μm(split 1:10)
Carrier gas	Hydrogen at 0.4 mL/min, constant flow
Gas for FID	Hydrogen at 70 kpa and air at 50 kpa
Injector temperature	340 °C
Detector temperature	340 °C
Temperature oven	320 °C; heat at 5 °C/min to 340 °C, maintain for 25 minutes

3.2.7.6 Identification of diacylglycerol isomers

The standards of dipamitin, distearin, dilaurin and diolein were prepared by adding 200 μL silylation reagent and then the test tube was sealed and stood for 20 minutes at room temperature. After silylation, 1 mL acetone was added. The test tube was left to stand for 10 minutes until the precipitate formed. The top clear solution was transferred to a GC vial before injection into the gas chromatograph. Using the retention time to identify the 1,2- and 1,3-diacylglycerols in the test portion.

3.2.7.7 Analysis of results

Calculate the percentage mass fraction, $\omega_{1,2}$, of 1,2-diacylglycerols as follows:

$$\omega_{1,2}=\frac{A_{1,2}}{\sum A}\times 100$$

Where

$A_{1,2}$ is the peak area of all 1,2-diacylglycerols (C_{32},C_{34},C_{36}) present in the test portion;

ΣA is the sum of the peak areas of the individual 1,2- and 1,3-diacylglycerols (C_{32},C_{34},C_{36}) (International Standard, 2009b).

3.3 Analysis of Data

All experiments and analytical determinations were carried out at least in duplicate.

Data analyses were performed using linear regression, mean value and standard deviation by using Microsoft Excel.

For zero order. The equations for calculating shelf life are: $\nabla = [X] - [X]_0 = kt$

For first order. The equations for calculating shelf life are:

$\nabla = \ln[X] - \ln[X]_0 = kt$

Where :

∇: the process effect

[X]: concentration after time t

$[X]_0$: concentration at zero time

k: reaction rate constant

t: time (day)

Chapter Four: Quality changing analysis of New Zealand extra virgin olive oil

4.1 Chemical changes in New Zealand extra virgin olive oil during storage

The consumption of olive oil has increased worldwide since consumers prefer to select least-processed foods (Patumi et al., 2003; Morelló et al., 2004). Therefore, high quality olive oil is increasingly in demand and the maintenance of the oil quality during the period between purchase and consumption is important (Morelló et al., 2004). Significantly, extra virgin olive oil's characteristics: such as aroma, taste, colour and nutritive properties make it easily distinguished from other edible vegetable oils (Morelló et al., 2004; Bendini et al., 2007). It is therefore a matter of great concern to preserve the product without loss of these positive attributes (Caponio et al., 2005).

Methods have been developed to evaluate the quality attributes and compare them with standards for extra virgin olive oil (Ramirez-Tortosa et al., 1999; Gandul-Rojas et al., 2000; Guimet et al., 2005; Sinelli et al., 2010). The procedures have been developed not only for the classification of the oil but also the identification of the adulteration of pure olive oil by soya oil, sunflower oil, corn oil, walnut oil and hazelnut oil and also for quality of stored olive oil (Christy et al., 2004; Cecchi et al., 2006).

As previously observed, there are a range of very strict quality parameters limits for EVOO. The first stage of this project was to determine the chemical changes in two

varieties of New Zealand extra virgin olive oil during six months storage at four temperatures (7 °C, 20 °C, 30 °C and 40 °C).

The cultivars of EVOO examined in this research were 'Frantoio' and 'Leccino' which are Italian cultivars. 'Frantoio' shows a high and constant productivity and adaptation capacity to different agronomical conditions; although cold-sensitive. While 'Leccino' is cold tolerant with an early start and high yield, since a low resistance to fruit drop has been described, as well as tolerance against different plant diseases (Aguilera et al., 2005).

The two cultivars EVOO were of high quality, having low values of free fatty acids and peroxide value before testing. The basic indices were shown in Table 9.

Table 9. Quality parameters for the storage trial EVOO (n=3).

	Free Fatty Acids (% $^{w}/_{w}$ oleic acid)	Peroxide value (meqO_2/kg)
'Frantoio'	0.2 ± 0.3	4.9 ± 1.2
'Leccino'	0.2 ± 0.4	4.9 ± 0.3

The stored olives oils were monitored for changes in peroxide value, K values, total phenolic content, bitterness index, percentage of pyropheophytin a formed and the ratio of 1,2-diacylglycerol to total diacyglycerol. To determine if changes in these parameters were significant over the six month storage period, the changes were analysed with a statistical *t*-test to determine if changes were significant, as shown in Table 10.

4.2 Changes in peroxide value in extra virgin olive oil

Oxidative rancidity development has been recognized as the predominant cause of olive oil deterioration during storage (Morelló et al., 2004; Bešter et al., 2008). The extent of oxidation in olive oil has been frequently evaluated by measuring peroxide value (PV) to monitor the initial stage of oxidation (Satue et al., 1995; Bešter et al., 2008). The index is related to the hydroperoxides, the primary oxidation products, which are unstable and readily decompose to form mainly volatile aldehyde compounds (Satue et al., 1995). Because these compounds are directly responsible for rancid flavours, they are considered important markers of oxidative rancidity (Satue et al., 1995).

One of the most evident differences between the oils at the four different temperatures was the change of peroxide value. The peroxide value limit for EVOO should not exceed 20 meqO_2/kg as reported by all regulators (European Community regulation 2472/97, 1997; International Olive Council, 2010a; United States Standards for Grades of Olive Oil and Olive-Pomace Oil, 2010; Standards Australia, 2011). After six months storage, the peroxide value of the EVOO showed a distinct trend at the four temperatures for both varieties (Figure 12). To be more specific, oils from both varieties (‘Frantoio’ and ‘Leccino’) underwent a significant increase in peroxide value up to 50 meqO_2/kg at 40 °C after 100 days storage. While at 30 °C it gradually rose until the peroxide value slightly exceeded the limit of 20 meqO_2/kg after approximately 115 days. Compared to the peroxide values at 7 °C and 20 °C, which remained below the limit. Both the two varieties of the olive oil were much more stable with lower PVs when they were stored at 7 °C and 20 °C than stored at 30 °C and 40 °C. Few changes were observed in the first month. Samples during this period would still contain higher concentration of phenolic compounds. Oxidation normally proceeds slowly at the initial stage and then a sudden rise occurs in the oxidation rate (Velasco & Dobarganes, 2002). A statistical t-test for ‘Frantoio’ olive oil stored at

7 °C and 20 °C showed no significant difference ($p > 0.05$) (Table 10) between the Day 0 and the end of the storage period. Conversely, 'Leccino' showed significant difference ($p < 0.05$) at 7 °C and 20 °C between the Day 0 and the end of the storage period (Table 10). At 7 °C the PV maintained at around 4.8 meqO_2/kg and it increased from 4.87 to 5.70 meqO_2/kg at 20 °C.

To determine if there was a significant difference for each quality index tested at the end of the storage period, t-tests were performed on the data values to determine significance. These results are shown in Table 10.

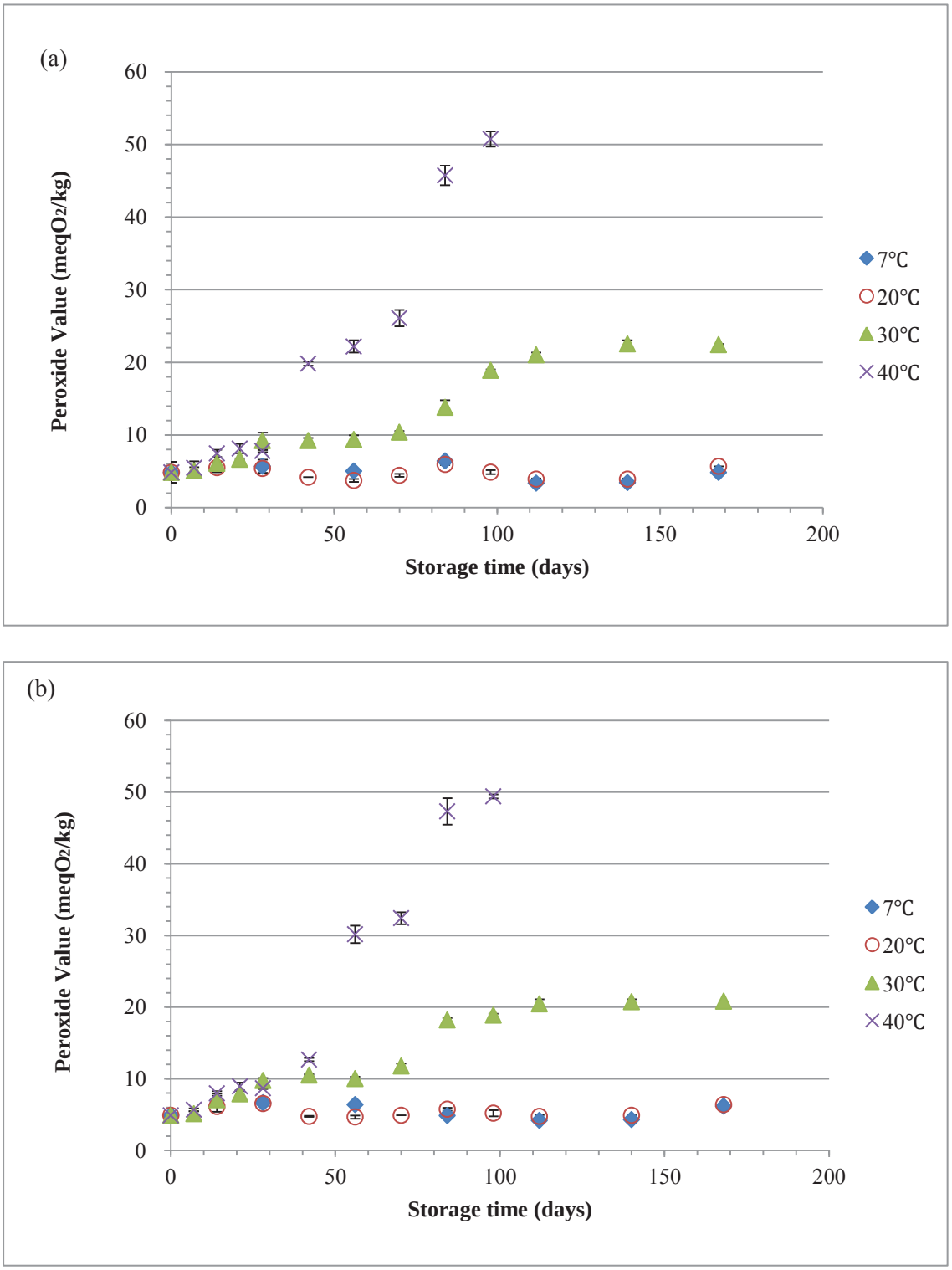


Figure 12. Changes of peroxide value (meqO_2/kg) in extra virgin olive oils of (a) 'Frantoio' and (b) 'Leccino' variety during storage at 7 ℃, 20 ℃, 30 ℃ and 40 ℃. Data plotted are the mean values. Error bars are the standard deviation (n=2 - 5).

Table 10. A statistic t-Test for the changes of pyropheophytin a %, peroxide value, total phenolics, bi

1,2-diacylglycerol ratio during storage at 7, 20, 30 and 40 °C. Results for each test from Day 0 and the l

			Day 0	Last day of storage (mean value)	No. o sto
Pyropheophytin a %	‘Frantoio’	7 °C	0 ± 0	0 ± 0	1
		20 °C		3.37 ± 0.61	1
		30 °C		19.56 ± 0.06	1
		40 °C		45.82 ± 2.72	1
	‘Leccino’	7 °C	0 ± 0	0.68 ± 0.16	1
		20 °C		3.33 ± 0.01	1
		30 °C		18.34 ± 0.49	1
		40 °C		38.81 ± 1.25	1
Peroxide value	‘Frantoio’	7 °C	4.87 ± 1.06	4.85 ± 0.07	1
		20 °C		5.70 ± 0	1
		30 °C		22.45 ± 0.07	1
		40 °C		50.75 ± 1.06	9
	‘Leccino’	7 °C	4.88 ± 0.39	6.15 ± 0.21	1
		20 °C		6.40 ± 0	1
		30 °C		20.8 ± 0	1
		40 °C		49.40 ± 0.28	9

Total Phenolics	‘Frantoio’	7 °C	1005 ± 124	497 ± 31	1
		20 °C		511 ± 25	1
		30 °C		456 ± 36	1
		40 °C		341 ± 24	1
	‘Leccino’	7 °C	1069 ± 279	547 ± 12	1
		20 °C		450 ± 13	1
		30 °C		355 ± 13	1
		40 °C		430 ± 38	1
Bitterness index	‘Frantoio’	7 °C	5.76 ± 0.2	5.02 ± 0.02	1
		20 °C		4.50 ± 0.07	1
		30 °C		4.27 ± 0.05	1
		40 °C		4.42 ± 0.08	1
	‘Leccino’	7 °C	5.72 ± 0.05	4.95 ± 0.1	1
		20 °C		4.87 ± 0.03	1
		30 °C		4.65 ± 0.07	1
		40 °C		4.31 ± 0.04	1

1,2-diacylglycerol ratio	‘Frantoio’	7 °C	92.87 ± 1.05	91.12 ± 0.85	1
		20 °C		91.03 ± 0.3	1
		30 °C		64.19 ± 0.4	1
		40 °C		56.52 ± 0.09	9
	‘Leccino’	7 °C	92.57 ± 0.23	90.55 ± 0.19	1
		20 °C		80.70 ± 0.22	1
		30 °C		62.59 ± 0.06	1
		40 °C		52.53 ± 0.14	9

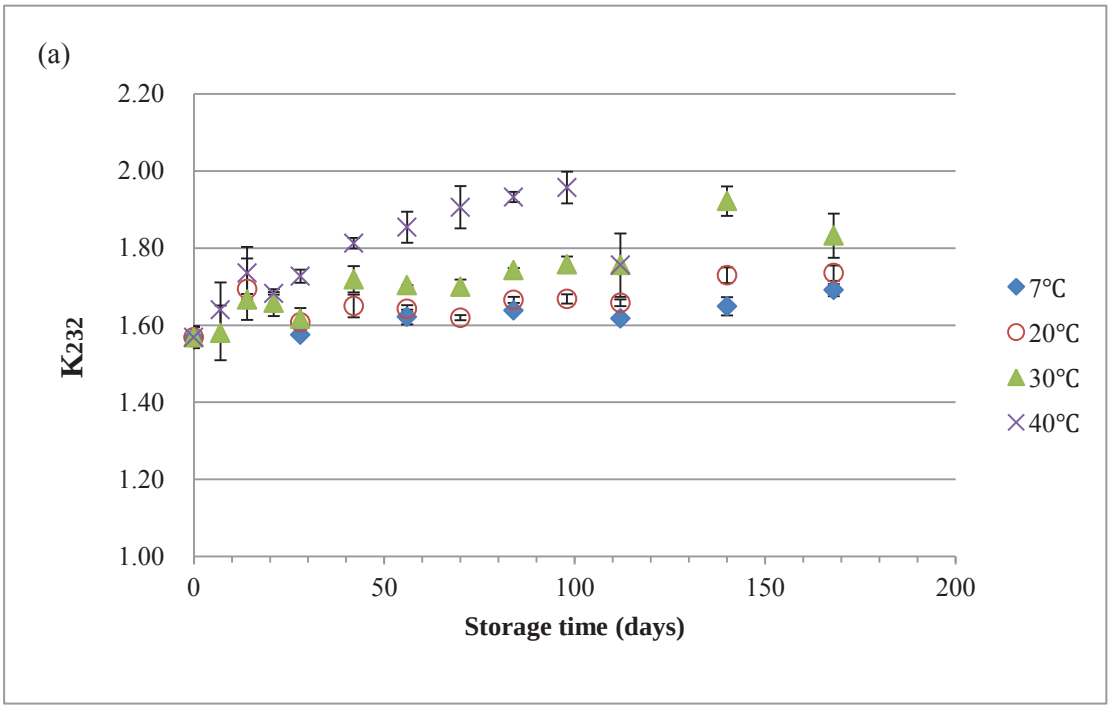
K_{232}	‘Frantoio’	7 °C	1.57 ± 0.03	1.69 ± 0.02	1
		20 °C		1.74 ± 0.02	1
		30 °C		1.83 ± 0.06	1
		40 °C		2.05 ± 0.04	1
	‘Leccino’	7 °C	1.59 ± 0.04	1.68 ± 0.02	1
		20 °C		1.76 ± 0.02	1
		30 °C		1.82 ± 0.03	1
		40 °C		1.87 ± 0.03	1
K_{270}	‘Frantoio’	7 °C	0.09 ± 0.03	0.14 ± 0.02	1
		20 °C		0.15 ± 0.01	1
		30 °C		0.15 ± 0	1
		40 °C		0.17 ± 0.01	1
	‘Leccino’	7 °C	0.06 ± 0.01	0.14 ± 0.01	1
		20 °C		0.14 ± 0	1
		30 °C		0.16 ± 0.01	1
		40 °C		0.18 ± 0	1

Note: ND= No difference; SD= Significantly different; NSD= No significant differences.

4.3 Changes of the Specific Extinction Coefficients K_{232} and K_{270} in extra virgin olive oil

Similar to peroxide value, the measure of extinction coefficients K_{232} and K_{270} indicates the degree of oxidation which has occurred in the oil. K_{232} is a measure of the primary oxidation products, conjugated dienes, which are formed by a shift in one of the double bonds (Cecchi et al., 2006; Bešter et al., 2008). K_{270} is increased by conjugated trienes (the primary oxidation products of linolenic acid) and secondary products of oxidation, such as aldehydes and ketones (Cecchi et al., 2006; Bešter et al., 2008).

The maximum limit for K_{232} is 2.50, 0.20 for K_{270} and 0.01 for ΔK, for an extra-virgin olive oil (European Community regulation 2472/97, 1997; the International Olive Council, 2010a; United States Standards for Grades of Olive Oil and Olive-Pomace Oil, 2010; Standards Australia 2011). Figure 13 and Figure 14 show that both of the two varieties of olive oils showed increased spectrophotometric parameters during storage. The increase of K_{232} was greater than the increase of K_{270}. This difference between K_{232} and K_{270} are partially in disagreement with data reported by Gutierrez & Fernandez (2002) who showed the K_{232} coefficient increased in oils stored at 30 °C, although the limit of 2.50 fixed for the extra virgin quality was not exceeded during the storage. While the K_{270} value also registered a sharp increase in oils stored at 30 °C. For the high storage temperatures in this trial, the K values increased at a faster rate, showing a similar trend to the peroxide value which can be partially explained by the peroxide degradation. Nevertheless, all of the data were below the maximum limit values. The statistical *t*-test showed significant difference ($p < 0.05$) (Table 10) for both K_{232} and K_{270} between day 0 and the last day of storage. However, no ΔK can be detected throughout the storage period.



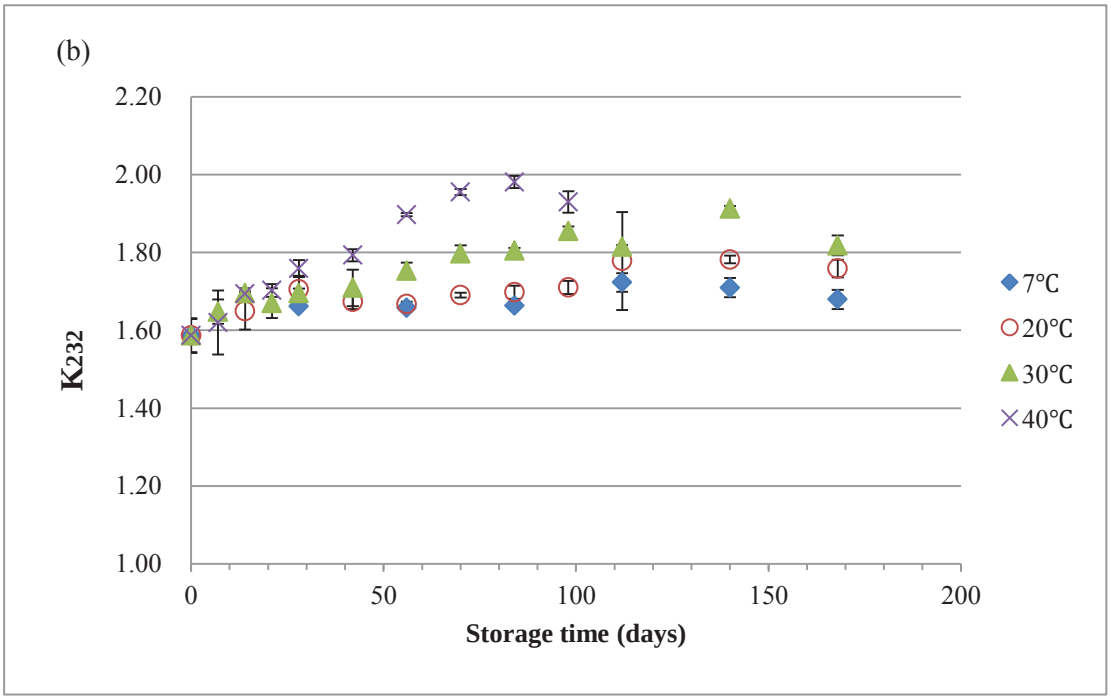


Figure 13. Changes of K_{232} in extra virgin olive oils of (a) 'Frantoio' and (b) 'Leccino' variety during storage at 7 ℃, 20 ℃, 30 ℃ and 40 ℃. Data plotted are the mean values. Error bars are the standard deviation (n=2).

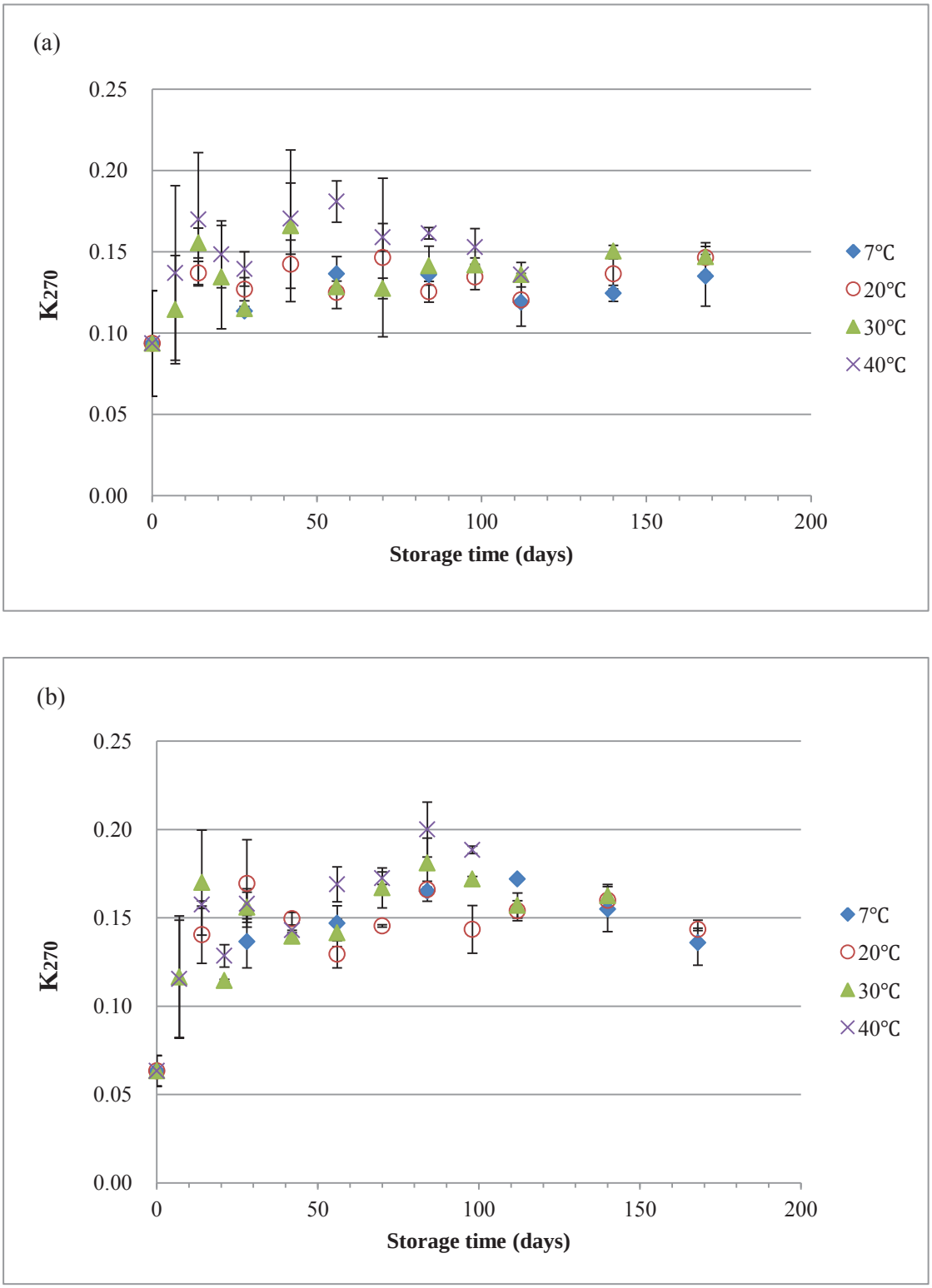
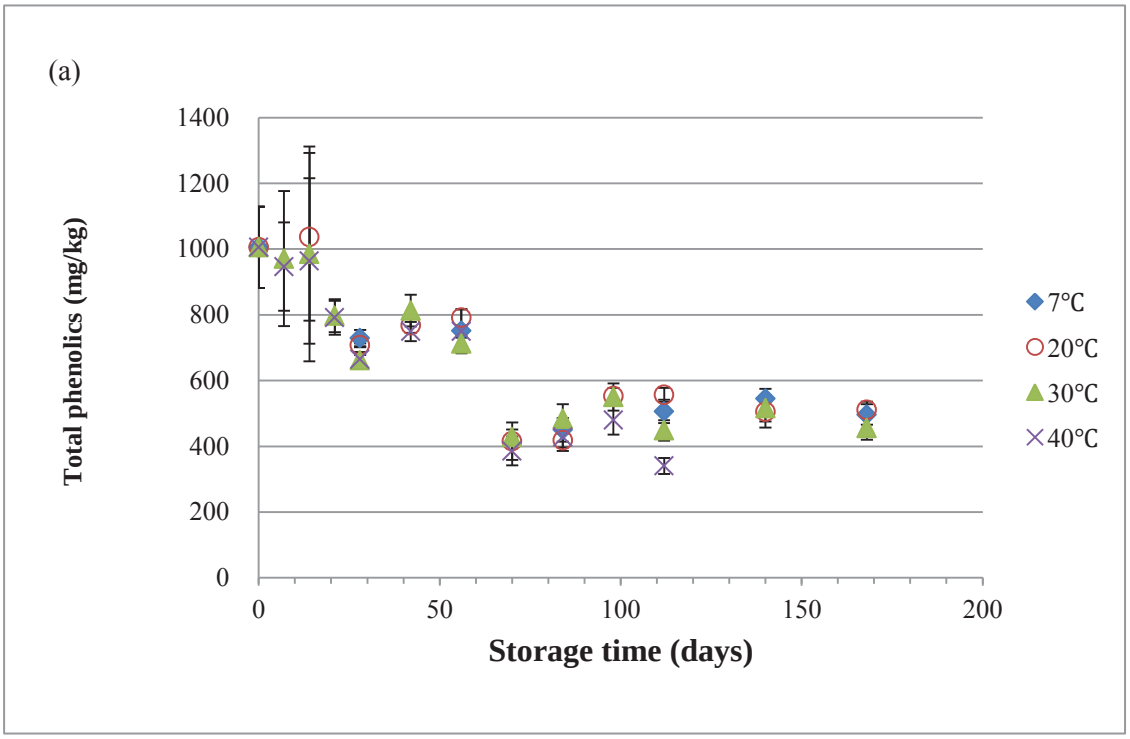


Figure 14. Changes of K_{270} in extra virgin olive oils of (a) 'Frantoio' and (b) 'Leccino' variety during storage at 7 ℃, 20 ℃, 30 ℃ and 40 ℃. Data plotted are the mean values. Error bars are the standard deviation (n=2).

4.4 Changes of total phenolic in extra virgin olive oil

Oxidative rancidity development has been recognised as the predominant cause of oil deterioration during storage (Morelló et al, 2004). This is a reaction between unsaturated fatty acids, regardless of whether they are in their free state or esterified as a triglyceride molecule and oxygen. It is also referred to as autoxidation because the activation energies of the first two reaction steps are very low. Therefore, autoxidation in oils can neither be prevented by maintaining cool storage conditions nor by the exclusion of light (Kilcast & Subramaniam, 2000; Morelló et al, 2004). While numerous studies have demonstrated that phenolic compounds in olive oil play an important role in scavenging of free radicals which are produced by oxidation. (Servili et al., 2009; Bubonja-Sonje et al., 2011). EVOO is rich in phenolic compounds, which act as potent antioxidants, free radical scavengers and modulators of various oxygen dependent enzymes (Visioli et al., 2005).

Storage time had a significant effect on the total phenolic content in the EVOO. Specifically, both EVOO of 'Frantoio' and 'Leccino' experienced dramatically decreases at the four different temperatures from over 1000 mg/kg down to around 500 mg/kg (Figure 15), a significant loss of total phenolics occurred during storage (Table 10). For 'Leccino', the total phenolic content was maintained at around 500 mg/kg for the last three months of storage at 7 °C, while the phenolic content decreased to below 400 mg/kg during storage at 30 °C and 40 °C. These results are in agreement with previous paper by Gutierrez & Fernandez (2002) when oxidation took place under accelerated conditions.



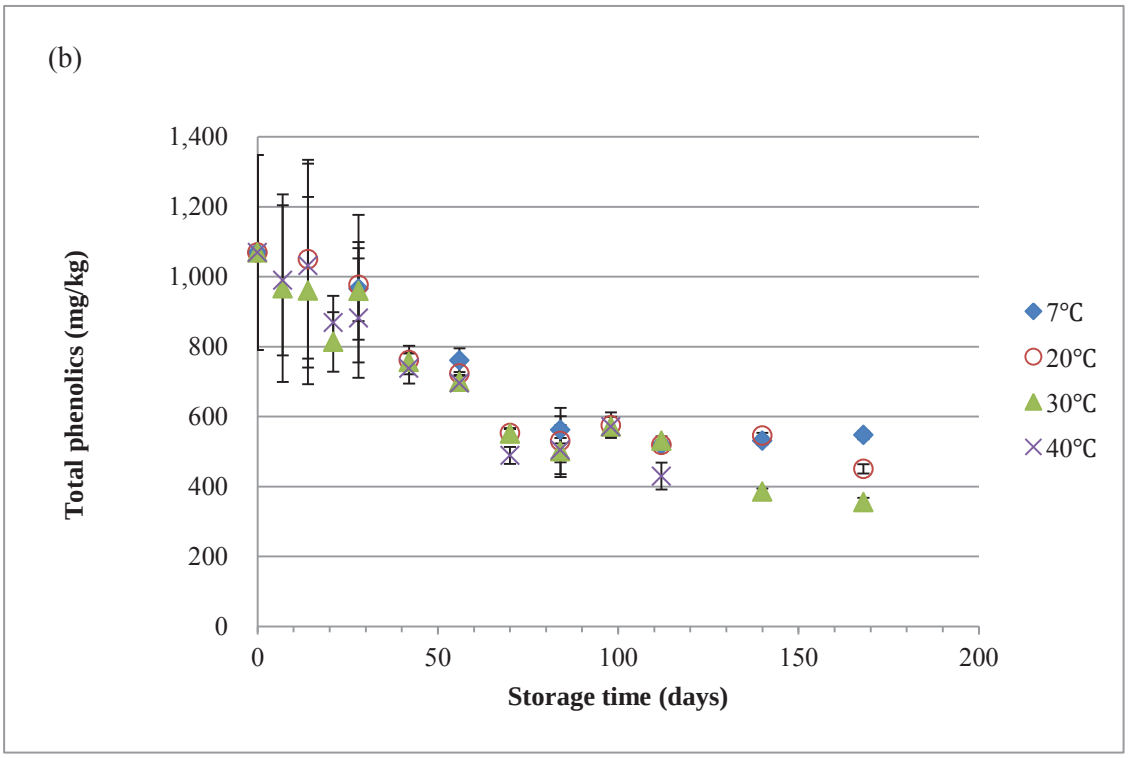


Figure 15. Changes of total phenolics (mg/kg) in extra virgin olive oils of 'Frantoio' and 'Leccino' variety during storage at 7 ℃, 20 ℃, 30 ℃ and 40 ℃. Data plotted are the mean values. Error bars are the standard deviation (n=2-3).

4.5 Changes of bitterness in extra virgin olive oil

Bitterness is generally considered a positive sensorial attribute of olive oil and enhances the overall flavour (Favati et al. 2013). It is often positively linked to the presence of phenolic compounds in EVOO (Siliani et al. 2006; Favati et al. 2013).

Bitter index values followed the similar pattern as total phenol content for 'Frantoio' (Figure 16a). For 'Leccino' olive oil the bitterness was found to be significantly less after storage but did not change as dramatically as the 'Frantoio' olive oil (Table 10, Figure 16b) In fact, it is generally accepted that the phenolic compounds of virgin olive oil are mainly responsible for the bitter attribute (Morelló et al, 2004; Bendini et al., 2007). The amount of phenolic compounds is an important factor when evaluating the quality of virgin olive oil, because of their involvement in its resistance to oxidation and its sharp bitter taste (Morelló et al, 2004). There were small differences for the bitterness values at the four different temperatures.

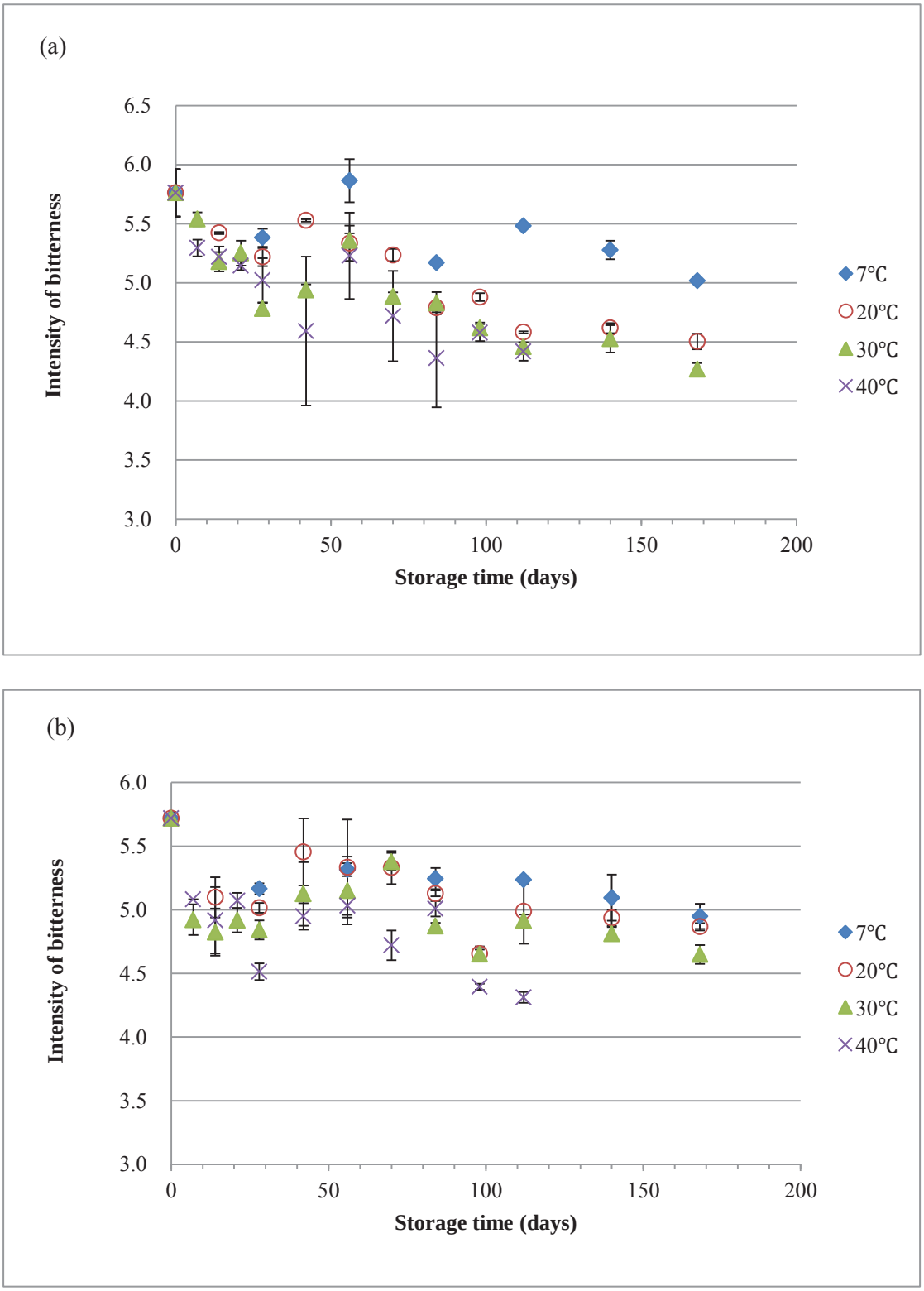


Figure 16. Changes of intensity of bitterness in EVOO of (a) 'Frantoio' and (b) 'Leccino' variety during storage at 7 ℃, 20 ℃, 30 ℃ and 40 ℃. Data plotted are the mean values. Error bars are the standard deviation (n = 2-4).

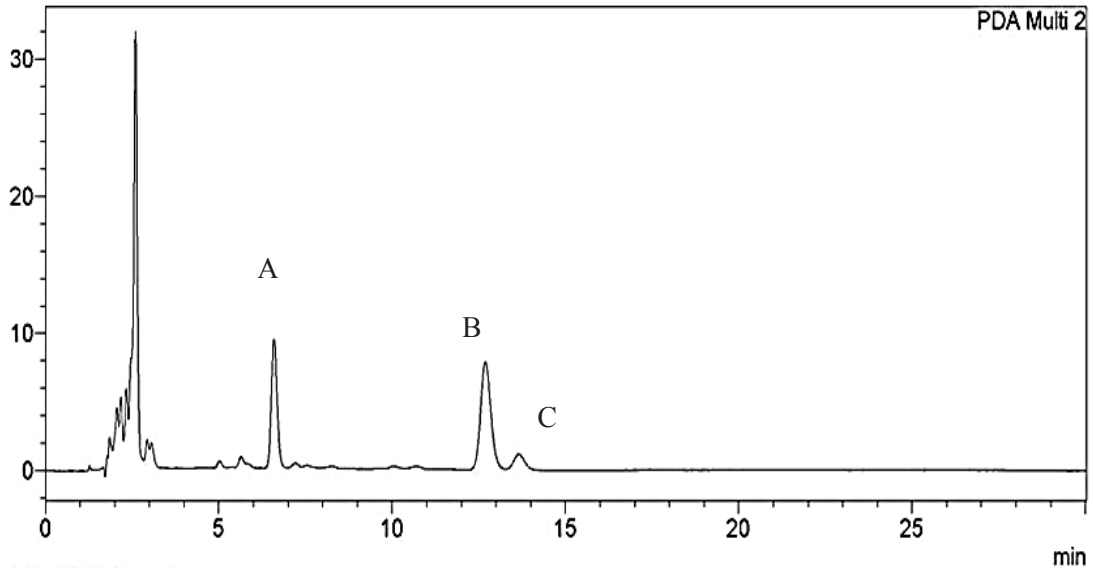
4.6 Changes of pyropheophytin in extra virgin olive oil determined by HPLC.

Chlorophylls play a mildly protective (antioxidant) role on the stability of olive oil (Boskou, 2010). When EVOO is stored, a new reaction occurs that is the formation of pyropheophytin. Thus, as it is a compound that is not produced during the oil extraction process, its formation over time was revealed as a useful parameter for monitoring the oxidative deterioration and loss of freshness in storage, allowing insight into the history of the EVOO (Aparicio-Ruiz et al., 2012). It has been reported that the formation of pyropheophytin which is one of the pheophytin a degradation products can occur during extended storage at low temperature (Anniva et al., 2006)

In Figure 17 (ii), an oil sample was processed at 160 °C for 60 minutes showing only the presence of pyropheophytin a and no B and C, which indicated D was the pyropheophytin a.

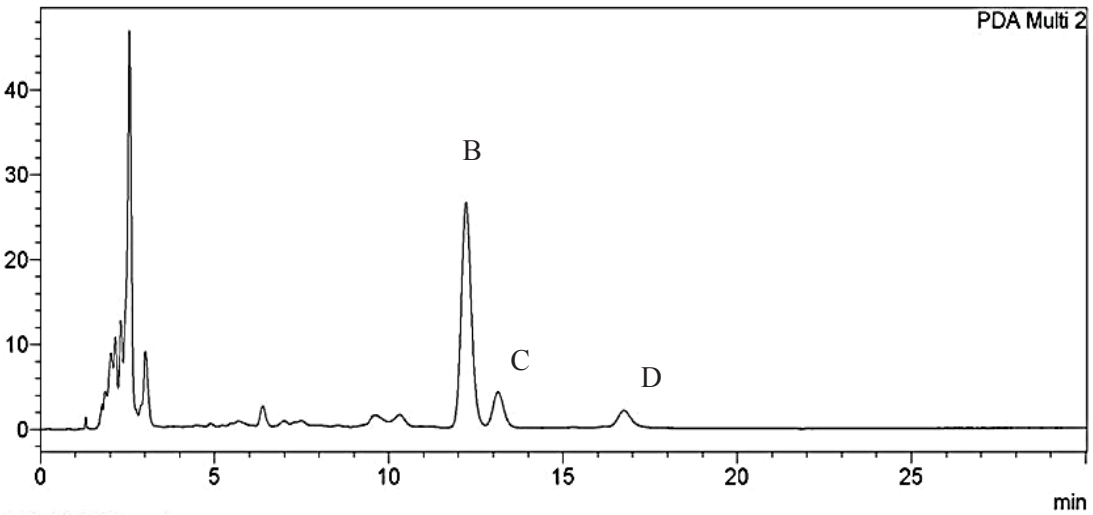
The Australian Standards (Standards Australia, 2011) have set a limit for pyropheopytin a (PPPs) (%) as equal to or less than 17%. Figure 18 shows the percentage of pyropheophytin relative to total chlorophyll content for the two cultivars at the four storage temperatures. It is shown that the percentage of pyropheophytin dramatically increased as the storage temperature increased. The pyropheophytin concentration was much more stable when samples were stored at low temperature. The values were within the Australian Standard's limit after storage at 7 and 20 °C for both cultivars.

(i)



Time (min)

(ii)



Time (minutes)

Figure 17. Typical high performance liquid chromatography of olive oil. A: Cholorophyll a; B: Pheophytin a; C: Pheophytin a'; D: Pyropheophytin a. (i): fresh extra virgin olive oil; (ii): stored extra virgin olive oil.

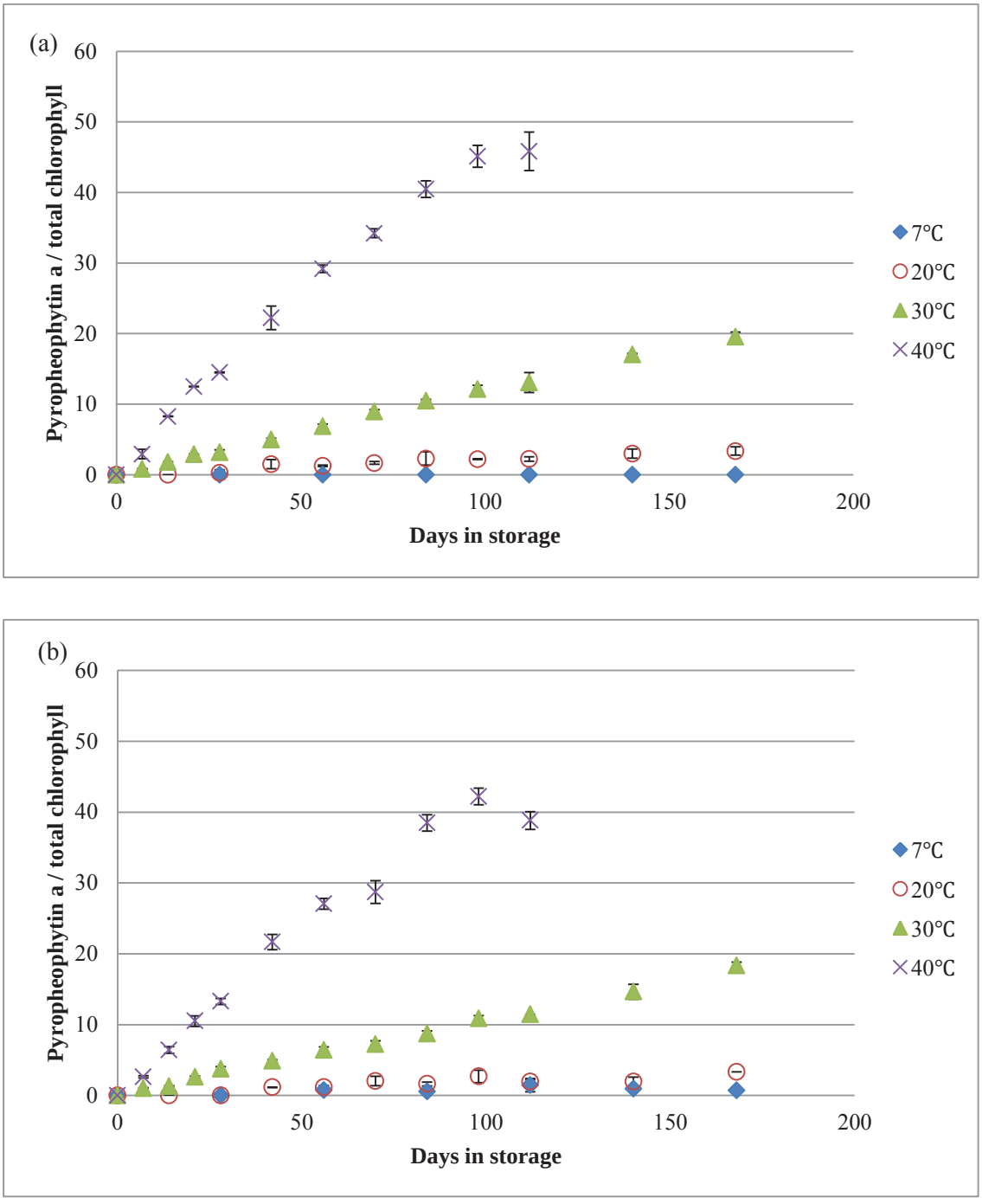


Figure 18. Changes in percentage of pyropheophytin a of total chlorophyll [% peak area of pyropheophytin/(peak area of chlorophyll, pheophytin a and pheophytin a')] in EVOO of (a) 'Frantoio' and (b) 'Leccino' variety during storage at 7 ℃, 20 ℃, 30 ℃ and 40 ℃. Data plotted are the mean values. Error bars are the standard deviation (n = 2-4).

4.7 Changes of 1,2-diacylglycerols in extra virgin olive oil by GC

Diacylglycerols are generated by acidic and enzymatic hydrolysis of triacylglycerols during transformation and storage of oils and fats. During the storage of oil, the changes in this fraction are due to an isomerization process (Pérez-Camino et al., 2001; Spyros et al., 2004; Narváez-Rivas et al., 2007). The 1,2-diacylglycerols spontaneously changes to 1,3-diacylglycerols since it is a more thermodynamically stable molecular species (Pérez-Camino et al., 2001; Spyros et al., 2004; Narváez-Rivas et al., 2007). In olive oil, the 1,3-/1,2-diacylglycerol ratio has proved to be useful for assessing the oil aging, conditions of storage, and handling (Spyros & Dais., 2000). Besides this, the kinetics of diacylglycerol formation allows the estimation of the storage time of virgin olive oil (Narváez-Rivas et al., 2007).

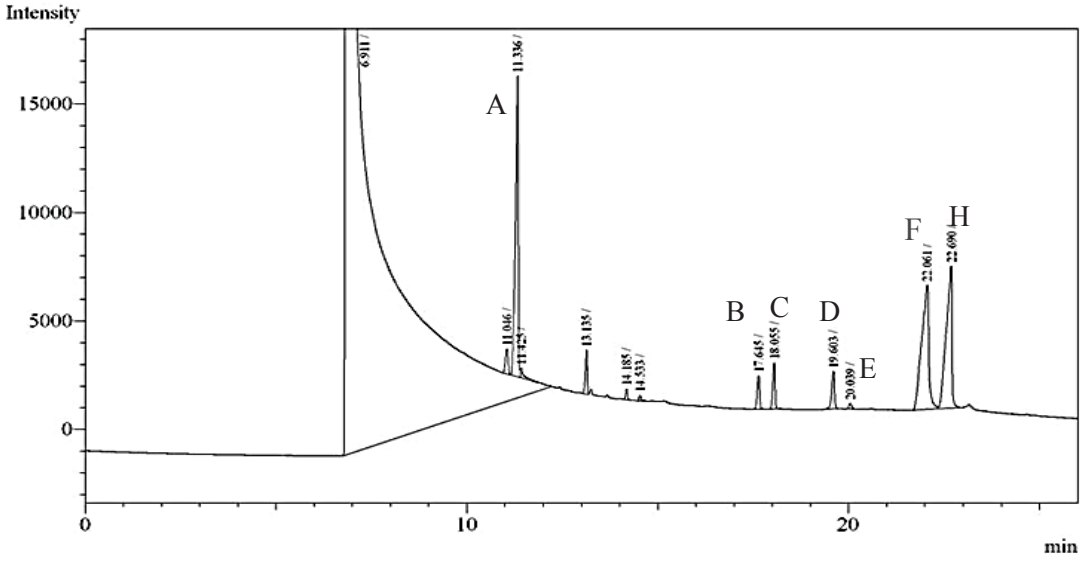


Figure 19. One typical gas chromatograph of a mixture of standards in EVOO.

A: dilaurin; B: 1,2-dipalmitin; C: 1,3-dipalmitin; D: 1,2-dilinolein; E: 1,3-dilinolein; F: 1,2-dioleoyl-sn-glycerol (1,2-diolein); H: 1,3-distearoyl-sn-glycerol(1,3-diolein).

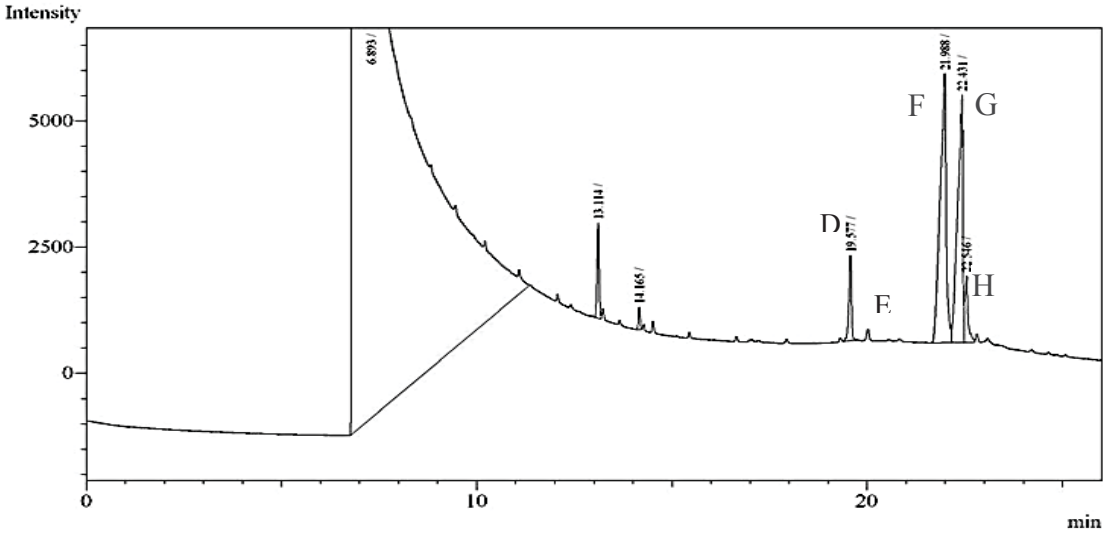


Figure 20. One typical gas chromatograph of mixture of one standard (1,2-distearoyl-sn-glycerol/1,2-distearoyl-rac-glycerol/1,2-distearin) and EVOO. D: 1,2-dilinolein; E: 1,3-dilinolein; F: 1,2-dioleoyl-sn-glycerol (1,2-diolein); G: (1,2-distearoyl-sn-glycerol/1,2-distearoyl-rac-glycerol/1,2-distearin); H: 1,3-distearoyl-sn-glycerol (1,3-diolein).

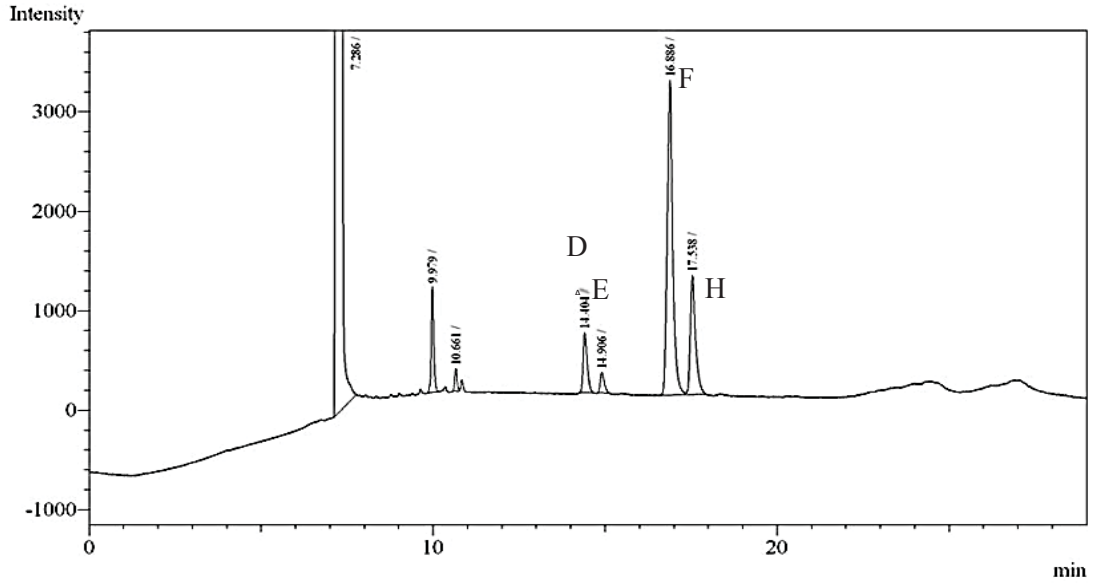


Figure 21. One typical gas chromatograph of EVOO.

D: 1,2-dilinolein; E: 1,3-dilinolein; F: 1,2-dioleoyl-sn-glycerol (1,2-diolein); H: 1,3-dioleoyl-sn-glycerol (1,3-diolein)

As shown in Figure 19, Figure 20 and Figure 21, an olive oil sample was mixed with four standards (dilaurin, dipalmitin, 1,2-distearoyl-rac-glycerol and dioleoylglycerol). Two peaks F and H were identified as 1,2-dioleoyl-sn-glycerol (1,2-diolein) and 1,3-dioleoyl-sn-glycerol (1,3-diolein) respectively. It was found by Visioli and Galli, (1998b) that olive oil has a unique fatty acid composition: its oleic acid content ranges from 56% to 84%, whereas linoleic acid constitutes 3 - 21%. Therefore, peak D and peak E were hypothesized as 1,2-dilinolein and E: 1,3-dilinolein, respectively.

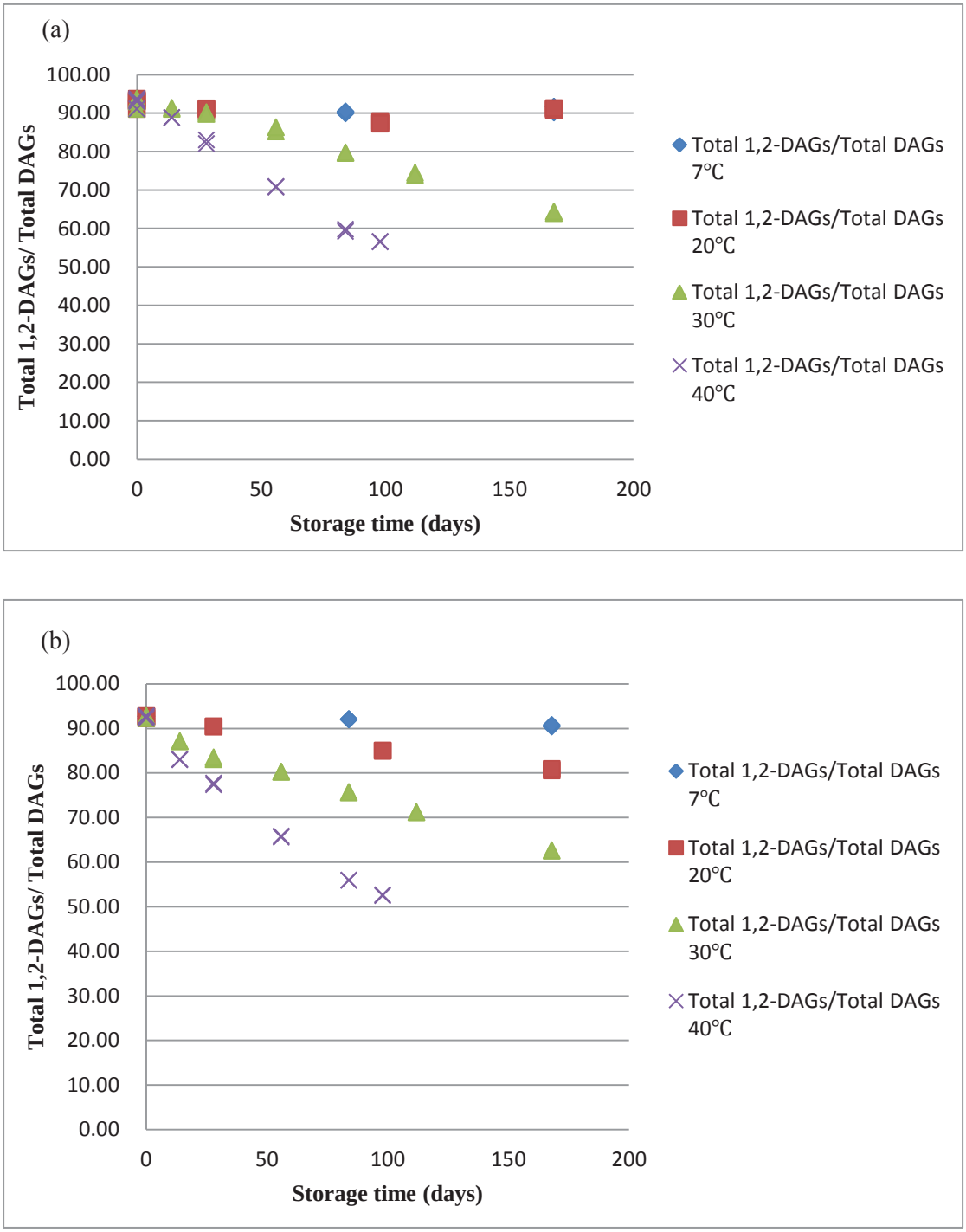


Figure 22. Changes in percentage of total 1,2-DAGs relative to total DAGs in extra virgin olive oils of (a) 'Frantoio' and (b) 'Leccino' variety during storage at 7 ℃, 20 ℃, 30 ℃ and 40 ℃. Data plotted are the mean values. Error bars are the standard deviation (n = 2-4).

For the diacylglycerols (DAGs), the Standards Australia (2011) has set the minimum limit for 1,2-DAGs as 35% (1,2-DAGs/Total DAGs). After 6 months storage, the conversion of 1,2-DAGs to 1,3-DAGs in EVOO was very similar in the two cultivars ('Frantoio' and 'Leccino'). Figure 22 shows the 1,2-DAG content maintained a high value at 7 °C (around 90%), whereas there was a noticeable decline in 1,2-DAGs when the temperature increased to 20 °C, 30 °C and 40 °C. The lowest percentage of 1,2-DAGs after six months storage was still above 50%. Fresh "just-obtained" olive oils from healthy olive fruits contain almost solely 1,2-DAGs (Pérez-Camino et al., 2001). As the literature review mentioned, 1,2-DAGs tend to isomerize to the more stable 1,3-DAG over time, which can give some information about the age of oil and storage conditions (Gunstone, 2004; Boskou et al., 2006a; Cossignani et al., 2007). The results agree with results presented in Fragaki et al. (2005) who reported that EVOO are characterized by high values of the ratio (1,2-DAGs/Total DAGs).

4.8 Discussion

From the above analytical results, the two cultivars ('Frantoio' and 'Leccino') of extra virgin olive oils prior to storage easily met premium quality indices of IOC, Australian and USA standards for peroxide value and K values. Also, the percentages for pyropheophytins (PPPs) and ratio of 1,2-diacylglycerols (1,2-DAGs) were within the limits set by the Standards Australia (2011). This indicated that the EVOO used in experiment was fresh and high quality. It is notable that there is no standard for the total phenolics and bitterness values as these values will depend on a number of factors including harvest time, processing method and climatic conditions.

The oils contained a high level of phenolic compounds and had low PVs at the beginning of storage, because the high phenolic concentration in olive oils are very effective in inhibiting antioxidant activity (Baldioli et al., 1996; Visioli & Galli, 1998a; Rotondi et al., 2004). There was observed a loss of phenolics with storage and

this was not found to be temperature dependent (Figure 15). As the oil aged, increases were found with PVs, K values and PPPs, whereas the following parameters decreased, total phenolic, bitterness index and 1,2-DAGs. It is notable that the oxidation process can be delayed by endogenous antioxidants that enhance the oxidative stability by preventing the propagation of lipid peroxidation or removing free radicals (Del Carlo et al., 2004). The results from the PV, K value, PPPs, bitterness and DAGs showed that the EVOO at the highest storage temperature (40 °C) oxidised faster than those at the lower three temperatures and had lower quality maintenance. Clearer results and trends were observed with the results for PPPs and DAGs compared to PV and K values.

Generally, the determination of quality of EVOO is mainly on the basis of the following parameters: free acidity, UV-absorption (K values), peroxide value, and sensory panel test score (Pérez-Camino et al., 2001). Both sensorial and chemical tests are required for the Australian, USA and IOC standards. Chemical tests monitor chemical processes in the oil, assessing oil quality, oxidation and any deterioration. In combination with chemical parameters, the sensory analysis should be performed according to the official method reported in Regulation (EC) 640 (2008). Sensory evaluation is an important tool to classify the oils in different quality and commercial categories (extra virgin, virgin, lampante). Such an official sensory evaluation completed by an accredited trained panel takes into account three attributes, that are characteristic (positive) for oils obtained from olives (1. fruity, perceived ortho- and retronasally being either “more green” or “more ripe”, 2. bitter and 3. pungent) and 5 main defects (rancid, fusty-muddy sediment, metallic, musty, winey), extended with a list of additional ones. The classification is granted if the standards for chemical analysis and sensory evaluation are met (Del Carlo et al., 2004; Bongartz & Oberg, 2011). However, there is a shortfall in this research as no sensory was carried out to assess if the oils were still acceptable. A trained sensory olive oil panel was not

available in New Zealand and it would have been too time-consuming and costly to develop and train a new panel for this research.

Chapter Five: Estimation of extra virgin olive oil shelf life using chemical indices

5.1 Rates of degradation reactions

Lipid oxidation occurs fairly slowly at room temperature. The oxidative stability of oil is difficult to define, but it is the main cause of olive oil quality deterioration and its reaction rate determines the shelf-life of this product (Gómez-Alonso et al., 2007; Krichene et al., 2010). Kinetic models were applied for the following two objectives: comparing stability of EVOO and predicting stability or shelf life (Pagliarini et al., 2000). EVOO can be consumed for many months after its production with no associated health risks, as it has the high stability under normal storage conditions (between 12 and 18 months) (Krichene et al., 2010; Aparicio-Ruiz et al., 2012). This chapter will deal with the oxidative stability and the shelf-life of EVOO determined at temperatures of 7, 20, 30 and 40 °C, under accelerated storage conditions.

5.1.1. Peroxide value

For the results from the EVOO storage trial, a kinetic study using the Arrhenius relationship was carried out for PV results.

Table 11. Values of the rate constant (k) at each storage temperature, the regression coefficient (R^2) and standard error for PV for zero order kinetics. (The graph was shown in Appendix 10.1)

Temperature	Rate constants for peroxide value increase			
	'Frantoio'		'Leccino'	
	k ($meqO_2kg^{-1}day^{-1}$)	R^2	k ($meqO_2kg^{-1}day^{-1}$)	R^2
40 °C	0.469 ± 0.027	0.928	0.484 ± 0.023	0.938
30 °C	0.123 ± 0.006	0.932	0.112 ± 0.006	0.918
20 °C	0.002 ± 0.003	0.021	0.001 ± 0.003	0.001
7 °C	0	0	0.005 ± 0.005	0.150

The rate constants for a zero order reaction are shown in Table 11 and the Arrhenius relationships for each cultivar are shown in Figure 23. There was no rate constant determined for 7 °C 'Frantoio' and no positive value on the regression analysis. Thus the rate constant k was 0. Based on regression analysis of the data at the four different temperatures during the storage, PV followed zero-order kinetics (R^2=0.9821) at all temperatures and for all samples studied. These results are in agreement with those reported by Calligaris et al. (2006) and Mancebo‐Campos et al. (2008).

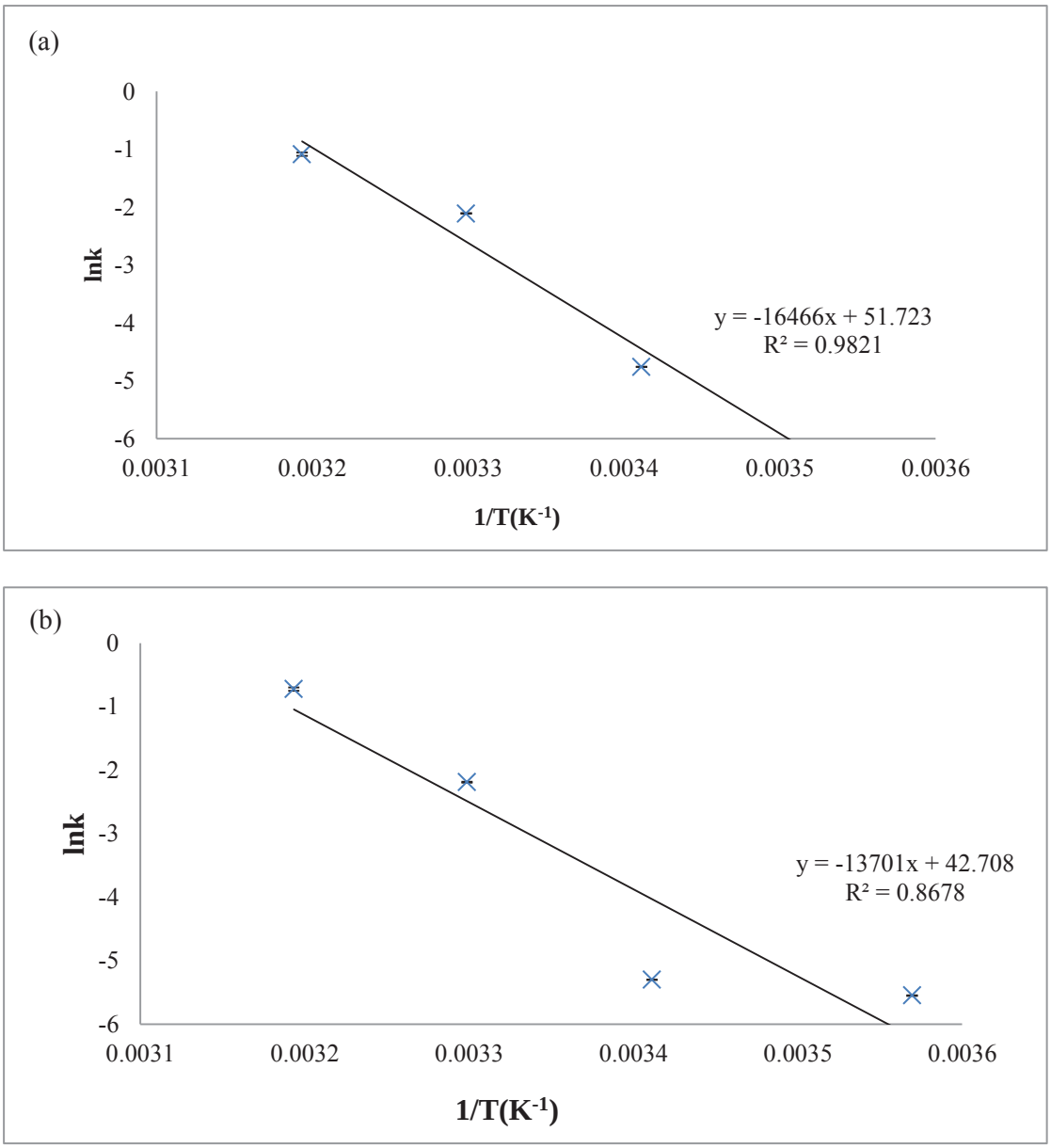


Figure 23. The kinetic behavior (zero order) of PVs affected by temperature for EVOO (a) 'Frantoio'; (b) 'Leccino'. Arrhenius plot conducted by regression analysis on all data (100 points) for rate constant (k) ± standard error.

5.1.2 Pyropheophytin

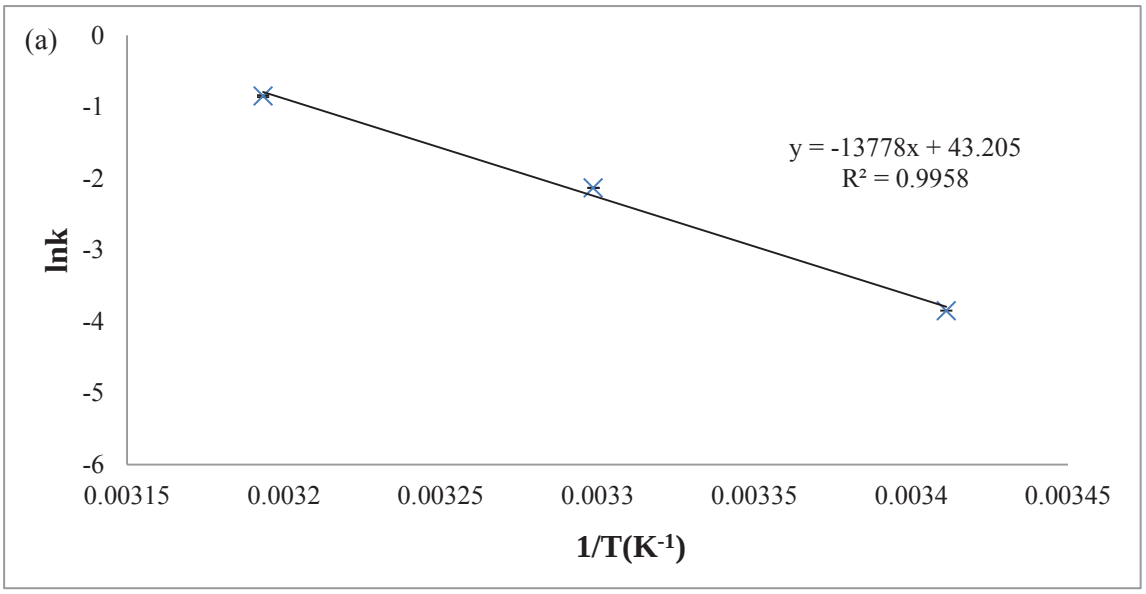
The pyropheophytin compound is not produced during the olive oil extraction process but during storage of EVOO, therefore a prediction of shelf life can be established following its kinetic behaviour (Aparicio-Ruiz et al., 2012). The chlorophyll

degradation follows this kinetic sequence:Chlorophyll $\xrightarrow{K_1}$ Pheophytin $\xrightarrow{K_2}$ Pyropheophytin (Schwartz & Elbe, 1983). There are also some publications on the kinetics of chlorophyll degradation (K_1) having been revealed that the rate of this degradation followed a first-order kinetic model (Van Loey et al., 1998; Ryan- Stoneham & Tong, 2000). Nevertheless, few publications were carried out on the pheophytin degradation or pyropheophytin formation (K_2). An old paper on pheophytin degradation showing it follows first order kinetics findings by Schwartz & Elbe (1983).

The first order and zero order kinetics for formation of pyropheophytin were both investigated in this research. The data analysis and Arrhenius plots are shown in Table 12, Figure 24, Table 13 and Figure 25, with the results indicating that zero order kinetics was a better predictor for shelf life than first order kinetics for the data collected based on correlation coefficients.

Table 12. Values of the rate constant (k) for the formation of pyropheophytin, the regression coefficient (R^2) and standard error for formation of pyropheophytin for zero order kinetics. (Graphs shown in Appendix 10.2)

Temperature	Rate constants for formation of pyropheophytin			
	'Frantoio'		'Leccino'	
	k (% day^{-1})	R^2	k (% day^{-1})	R^2
40 °C	0.427 ± 0.013	0.978	0.397 ± 0.016	0.964
30 °C	0.119 ± 0.002	0.993	0.105 ± 0.002	0.993
20 °C	0.021 ± 0.002	0.876	0.019 ± 0.002	0.763
7 °C	0	0	0.007 ± 0.002	0.374



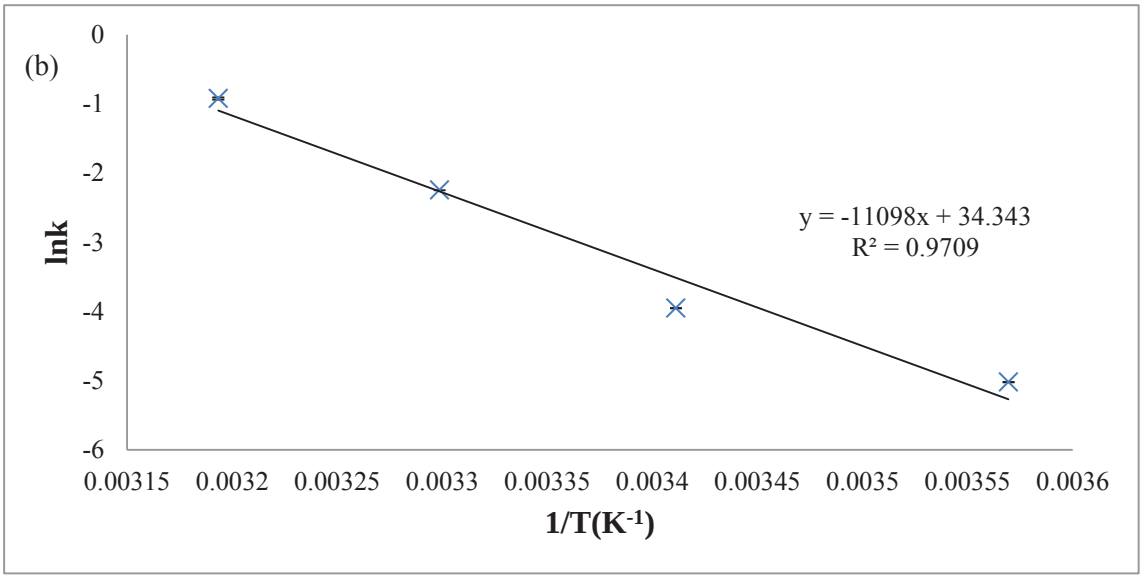


Figure 24. The kinetic behaviour (zero order) of pyropheophytin affected by temperature for EVOO (a) 'Frantoio'; (b) 'Leccino'.

Arrhenius plot was found by regression analysis on all data (100 points) for rate constant (k) ± standard error.

Table 13. Values of the rate constant (k), the regression coefficient (R^2) and standard error for PPP for first order kinetics. (Graphs shown in Appendix 10.3)

Temperature	Rate constants for formation of pyropheophytin			
	'Frantoio'		'Leccino'	
	k (day^{-1})	R^2	k (day^{-1})	R^2
40 °C	0.029 ± 0.003	0.765	0.030 ± 0.003	0.772
30 °C	0.018 ± 0.001	0.879	0.018 ± 0.001	0.873
20 °C	0.008 ± 0.001	0.854	0.007 ± 0.001	0.716
7 °C	0	0	0	0

The zero order rate constant for 'Frantoio' at 7 °C was 0 as there was found to be no difference between the day 0 value and the end of the storage date. Likewise, a positive rate constant value was not obtained when calculating first order kinetics for the values of 'Frantoio' and 'Leccino' at 7 °C.

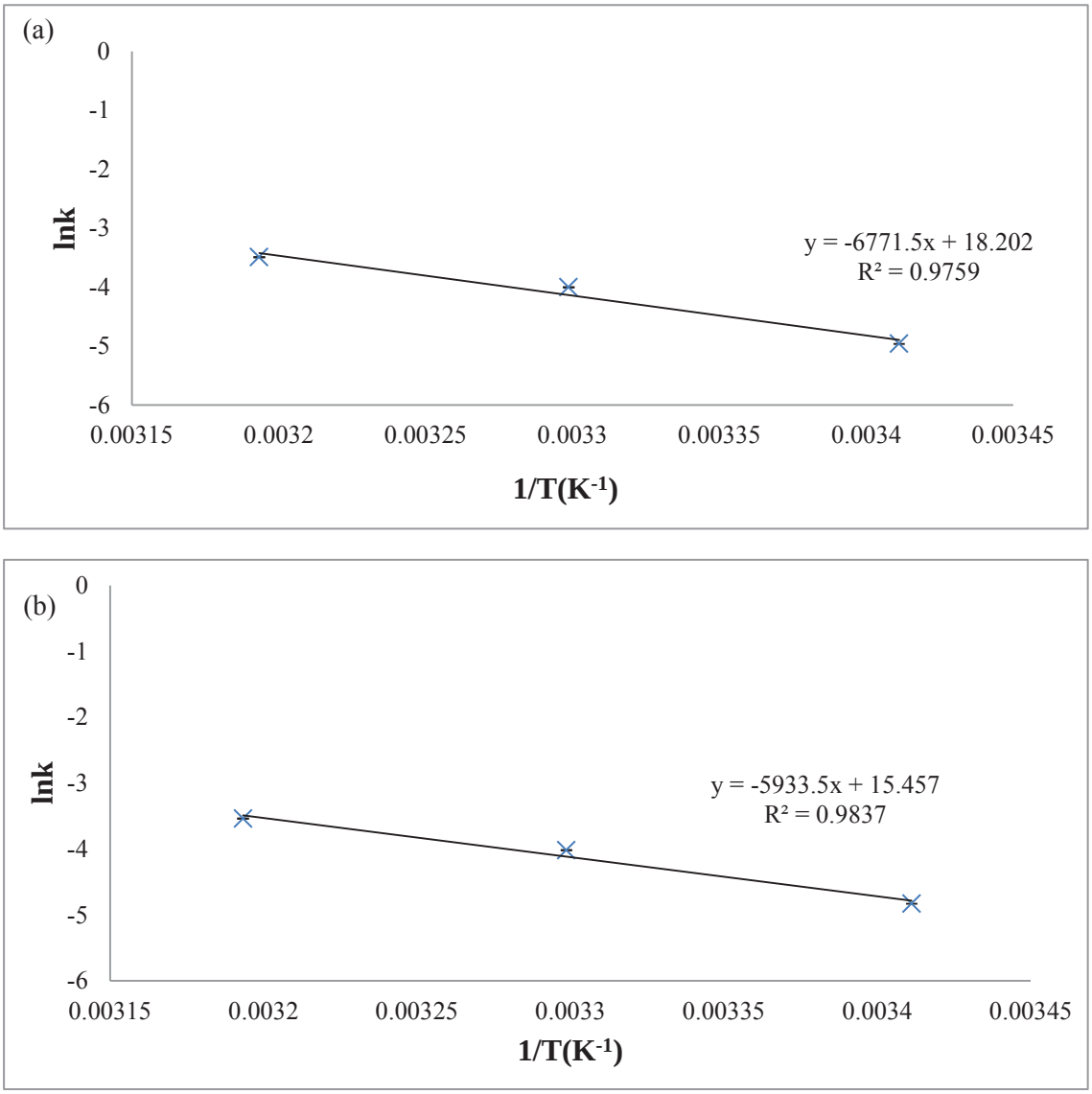


Figure 25. The kinetic behaviour (first order) of pyropheophytin increase affected by temperature for EVOO (a) 'Frantoio'; (b) 'Leccino'. Arrhenius plot was found by regression analysis on all data (100 points) for rate constant (k) ± standard error.

5.1.3 Diacylglycerols

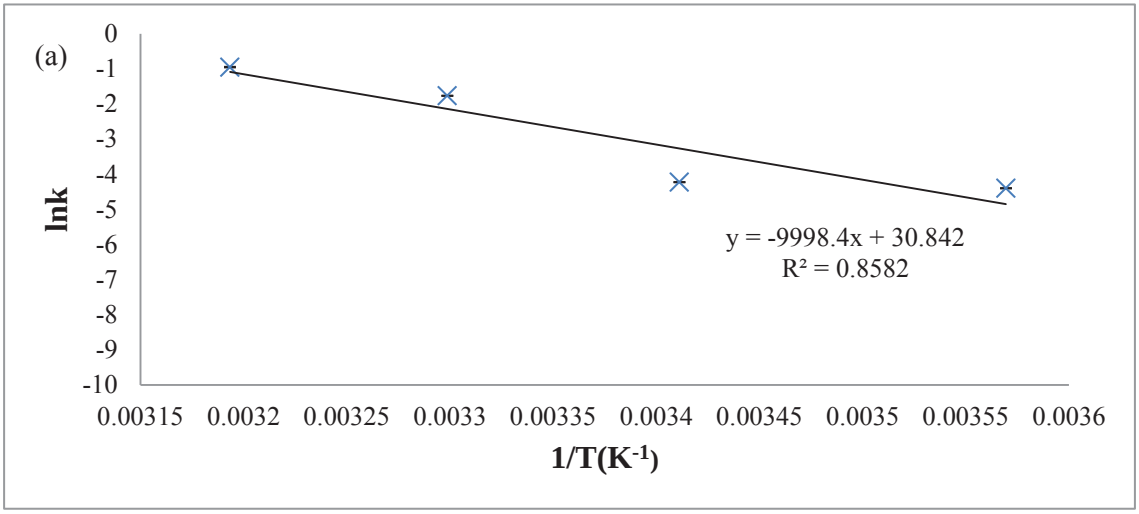
The DAG fraction can assume the significance of a valuable "fingerprint" of the virgin olive oil, because the ratios among DAG isomeric classes are influenced by the initial quality and by the storage of product (Cossignani et al., 2007). The storage temperature has a predominant role in the isomerization process (Paradiso et al.,

2012). Spyros et al. (2004) reported a kinetic study of DAGs formation and isomerization in fresh extra virgin olive oil samples of Greek origin with varying initial acidity and DAGs content. They investigated three different storage conditions: ambient temperature in the dark and in the light and at 5 °C in the dark. While in this research, a kinetic study of the ratio of 1,2-DAGs/Total DAGs in EVOO samples of two cultivars ('Frantoio' and 'Leccino') as a function of storage time under four temperatures 7, 20, 30 and 40 °C to predict the shelf life of the EVOO was conducted.

The data analysis and the illustration of zero and first order kinetics are shown in Tables 14 and 15 and Figures 26 and 27. At 20 °C the correlation coefficient for 'Frantoio' was very low as the ratio of 1,2-DAGs/Total DAGs fluctuate between 93.40% and 87.28%. It was found by Laszlo et al. (2008) DAG acyl kinetics was modelled employing a reversible first-order reaction scheme on the concentration of 1,2-DAGs and 1,3-DAGs, respectively. The ratio of 1,2-DAGs/Total DAGs was fitted to first order as shown in Figure 27.

Table 14. Values of the changing rate constant (k), the regression coefficient (R^2) and standard error for change in 1,2-DAGs/Total DAGs ratio for zero order kinetic. (Graphs are shown in Appendix 10.4).

Temperature	Rate constants for change in 1,2-DAGs/Total DAGs ratio			
	'Frantoio'		'Leccino'	
	k (% day^{-1})	R^2	k (% day^{-1})	R^2
40 °C	-0.386 ± 0.007	0.995	-0.411 ± 0.014	0.986
30 °C	-0.171 ± 0.005	0.988	-0.173 ± 0.007	0.978
20 °C	-0.015 ± 0.008	0.213	-0.072 ± 0.002	0.996
7 °C	-0.012 ± 0.005	0.355	-0.012 ± 0.001	0.911



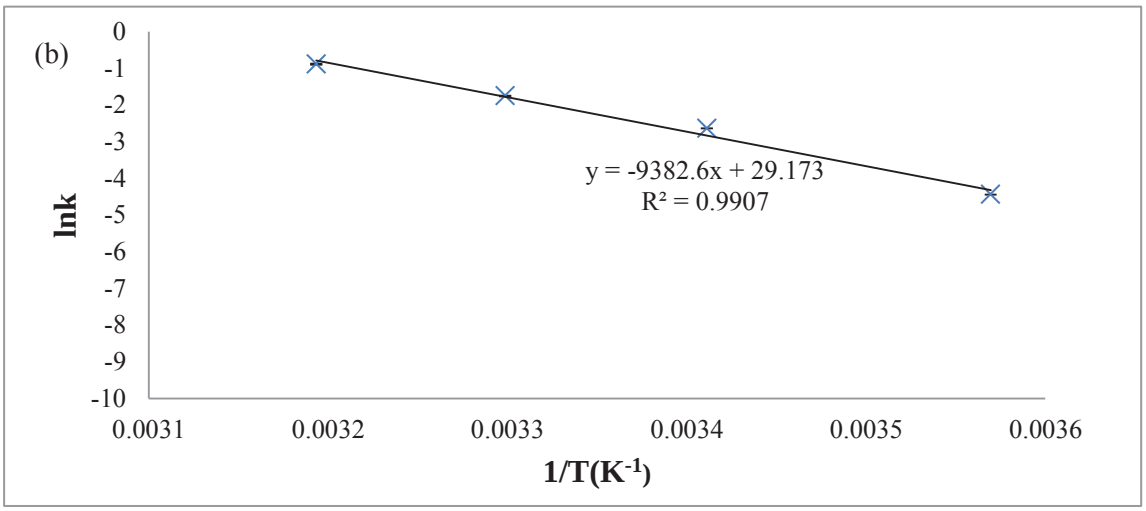
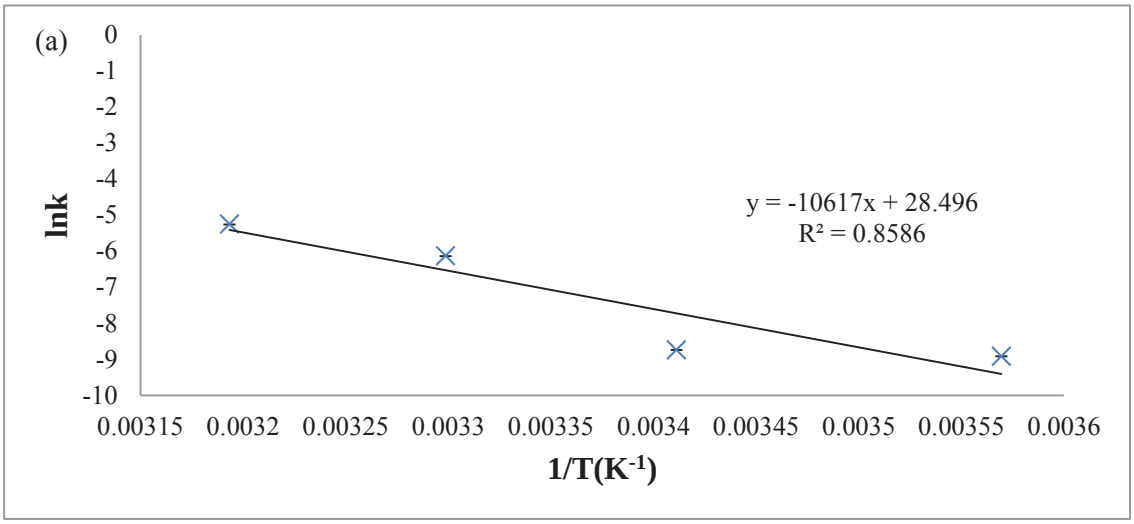


Figure 26. The kinetic behaviour (zero order) of 1,2-DAGs/Total DAGs influenced by temperature for EVOO (a) 'Frantoio'; (b) 'Leccino'.

Arrhenius plot found by regression analysis on all data (50 points) for rate constant (k) ± standard error.

Table 15. Values of the rate constant (k), the regression coefficient (R^2) and standard error for change in 1,2-DAGs/Total DAGs ratio for first order kinetic. (Graphs are shown in Appendix 10.5).

Temperature	Rate constants for change in 1,2-DAGs/Total DAGs ratio			
	'Frantoio'		'Leccino'	
	k (day^{-1})	R^2	k (day^{-1})	R^2
40 °C	$-5.2\times10^{-3} \pm 1\times10^{-4}$	0.994	$-5.8\times10^{-3} \pm 9\times10^{-5}$	0.997
30 °C	$-2.2\times10^{-3} \pm 8\times10^{-4}$	0.981	$-2.2\times10^{-3} \pm 7\times10^{-5}$	0.988
20 °C	$-1.6\times10^{-4} \pm 9\times10^{-4}$	0.208	$-8.3\times10^{-4} \pm 1\times10^{-5}$	0.998
7 °C	$-1.3\times10^{-4} \pm 6\times10^{-4}$	0.353	$-1.3\times10^{-4} \pm 1\times10^{-5}$	0.910



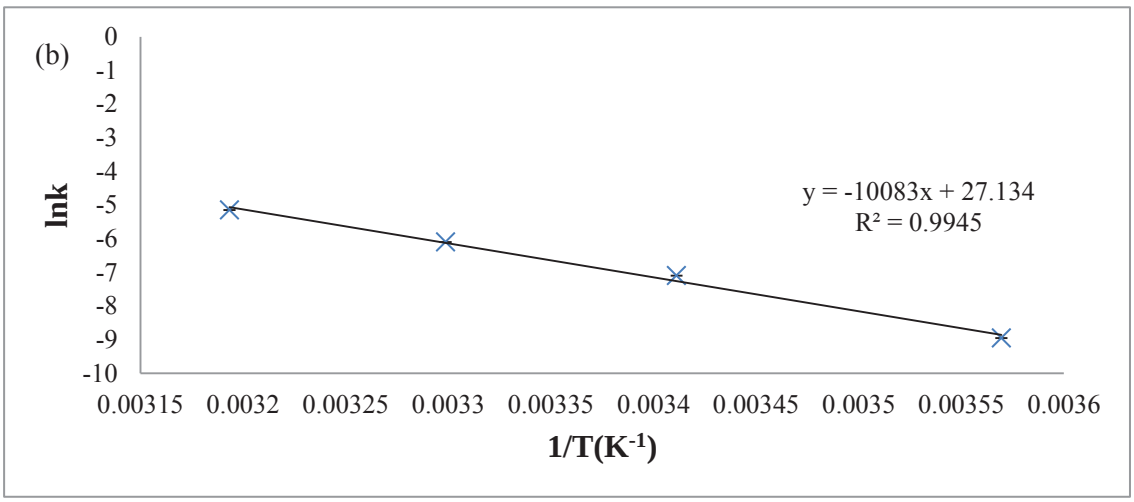


Figure 27. The kinetic behaviour (first order) of 1,2-DAGs/Total DAGs influenced by temperature for EVOO (a) 'Frantoio'; (b) 'Leccino'.

Arrhenius plot found by regression analysis on all data (50 points) for rate constant (k) ± standard error.

5.2 Shelf life estimation.

Based on the Arrenhius equations and kinetic models for peroxide value increase, pyropheophytin concentration increase and the change in the 1,2-DAG/Total DAG ratio and EVOO standard limits set by the Standards Australia (2011), the shelf life of

the EVOO was predicted for 10 °C and 20 °C. The results of the calculations are shown in Table 16, the examples of the calculation of shelf life are shown in Appendix 10.6.

Zero order kinetics was used to predict shelf life using PV and pyropheophytin%, first order for the change in 1,2-DAGs/Total DAGs ratio. The formation of pyropheophytin% was also found to follow first order kinetics, but with lower R^2 values hence shelf life based on pyropheophytin was predicted with zero order kinetics. Zero order and first order kinetics was used to predict shelf life for 1,2-DAGs/Total DAGs ratio.

The results obtained revealed that the shelf life of a high quality EVOO based on PV, the olive oil for 'Frantoio' can be stored for more than three years at 20 °C (in the dark) and more than two years for 'Leccino' (Table 16). It was calculated that they could be stored for more than 26 years at 10 °C (in the dark) and more than 12 years for 'Leccino'. These values are not realistic, because the oil would never last 26 years. Thus, other factors will also influence the shelf life and PV is not a good test for long term shelf life estimation. The shelf life based on the zero order kinetic of PPPs, the olive oil for 'Frantoio' could be stored for more than two years at 20 °C (in the dark) and more than 1.5 years for 'Leccino'. While 'Frantoio' can be stored for more than 11 years at 10 °C (in the dark) and more than 6 years for 'Leccino'. The shelf life based on the first order kinetic of 1,2-DAG/Total DAG ratio, the olive oil for 'Frantoio' can be stored for more than six years at 20 °C (in the dark) and around four years for 'Leccino'. Whereas, 'Frantoio' could be stored for more than 14 years at 10 °C (in the dark) and more than 8 years for 'Leccino'.

Table 16. Shelf life estimation based on kinetics of PV, PPP and DAGs at 10 °C and 20 °C.

		Estimated shelf life (years)			
Storage Temperature	Cultivar	Peroxide value	PPPs	DAGs	
		Zero order	Zero order	Zero order	First order
20 °C	'Frantoio'	3.6	2.1	4.2	6.1
10 °C		26.5	11.2	14.1	22.1
20 °C	'Leccino'	2.4	1.6	2.7	3.9
10 °C		12.4	6.1	8.5	13.0

5.3 Discussion

One objective of this research was to attempt to predict the shelf life for EVOO of good quality. The ideal test for measuring the changes in EVOO during storage would be a test ranging from 18 to 24 months, which implies that completing the oxidation process would take several years (Hrncirik & Fritsche, 2005). However, in these trials, samples were only monitored during the initial stage of the oxidation process.

From the data shown, a shorter shelf life was estimated based on the zero order kinetic of PPPs, with a slightly longer shelf life with first order kinetics of DAGs. Compared to PV and DAGs analysis, the PPPs analysis conducted gave a more sensitive detection, which indicated that PPPs could be a better indicator to identify the stored EVOO. Based on the most conservative estimate of shelf-life using pyropheophytin%, both olive oils had a shelf life of 1.5 to 2 years at 20 °C.

Free acidity was only monitored at time zero, whereas Spyros et al. (2004) found that the rate of the 1,2-DAGs to 1,3-DAGs isomerization increased with free acidity. GC analysis can be more accurate than the titration of free acidity.

There was no sensory evaluation carried out in this study, which may lead to the shelf life being a little shorter as generally the trained sensory panellists are more sensitive to off flavours. Olive oil generally has a relatively long shelf-life and only minor changes of sensory characteristics occur. Most producers consider 12-18 months as the maximum storage period from bottling to consumption (Morelló et al., 2004).

Generally, different cultivars of EVOO shelf life for storage at 10 and 20 °C are different. The cultivar of EVOO for ‘Frantoio’ can keep longer than ‘Leccino’, especially for the peroxide value and 1,2-DAGs parameter. It might be because other antioxidants such as Vitamin E are present in the oils to slow down the oxidation.

Chapter Six: Comparison of commercial olive and avocado oils.

6.1 Quality indices of commercial extra virgin olive oils

According to Zampounis (2006), the world olive oil consumption in the non-producing countries (France included) of the E.U. and the "Big New 5" (U.S.A., Australia, Canada, Japan, Brazil) has an annual rate of growth of 10.06% and 8.42% respectively. Their consumption has increased in the last 20 years, which already corresponds to 20% of world consumption. This development is mainly because the U.S. which has increased its consumption from 42 thousand tons in 1984 (after the International Olive Oil Council promotional activities started) to 248 thousand tons in 2004. World consumption increased almost one million tons (+54%) during the 20-year period under consideration. This means an annual compound rate of growth of 2.6%. García-González & Aparicio (2010) also reported that the production and consumption of olive oil are moving slowly but also moving beyond the Mediterranean countries, and olive trees are being planted in countries far from the Mediterranean basin such as in New Zealand and Argentina. Also, a large body of scientific evidence supports a move from the traditional Western diet, rich in saturated fat and sugars, to one similar to that consumed in the Mediterranean area, as being more healthful (García-González & Aparicio, 2010). Avocado oil has a composition closely resembling olive oil and can be directly substituted for it in a healthful cuisine (Birkbeck, 2002).

In this study, PV, free fatty acid, total phenolics, PPPs and DAGs were monitored in a number of oil samples to assess the quality of various oils available in New Zealand and some of which were imported EVOO.

6.1.1 Peroxide value for commercial oils

Various commercial EVOOs and avocado oil were tested for the peroxide values. The oils were stored at -80 °C after they were bought from supermarket. The results for the PVs are shown in Figure 28.

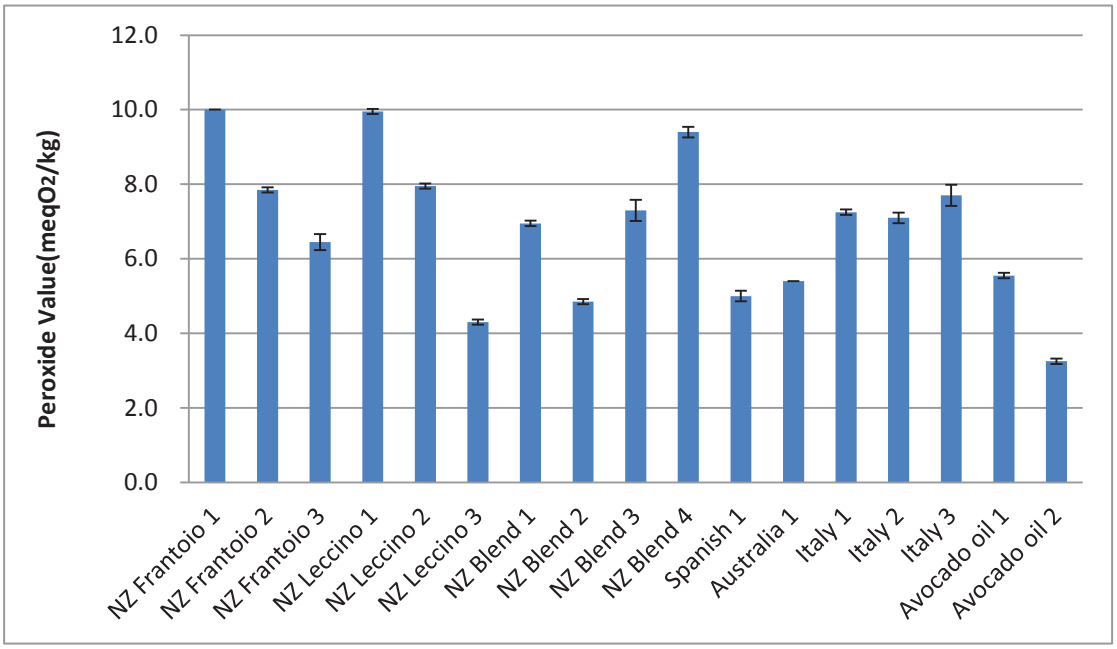


Figure 28. Comparison of peroxide value for commercial oils. (mean±standard deviation, n=3)

In Figure 28, the commercial EVOOs had low PV which was all below 10 $meqO_2/kg$, while the peroxide value limit for EVOO by International Olive Council (2010a) is 20 $meqO_2/kg$. More specifically, two varieties of New Zealand EVOOs (NZ 'Leccino' 3 and NZ Blend 2), Spanish 1, Australia1 and Avocado oil 2 had lower PV results compared to the other oils tested.

6.1.2 Free fatty acids for commercial oils

In Figure 29, it is shown that all of the EVOOs tested had free fatty acid levels below 0.8% $^{w}/_{w}$ oleic acid compared to the Avocado oil 1 tested which had higher values, Avocado oil 1 had a free fatty acid level of greater than 1%. The EVOO oil Australia

1 had a slightly higher free fatty acid percentage compared to the other commercial EVOOs. The red dotted line is shown in Figure 29 represents the limit for FFA% (w/w oleic acid) for EVOO according to IOC (2010a), United States Standards for Grades of Olive Oil and Olive-Pomace Oil (2010) and Standards Australia (2011).

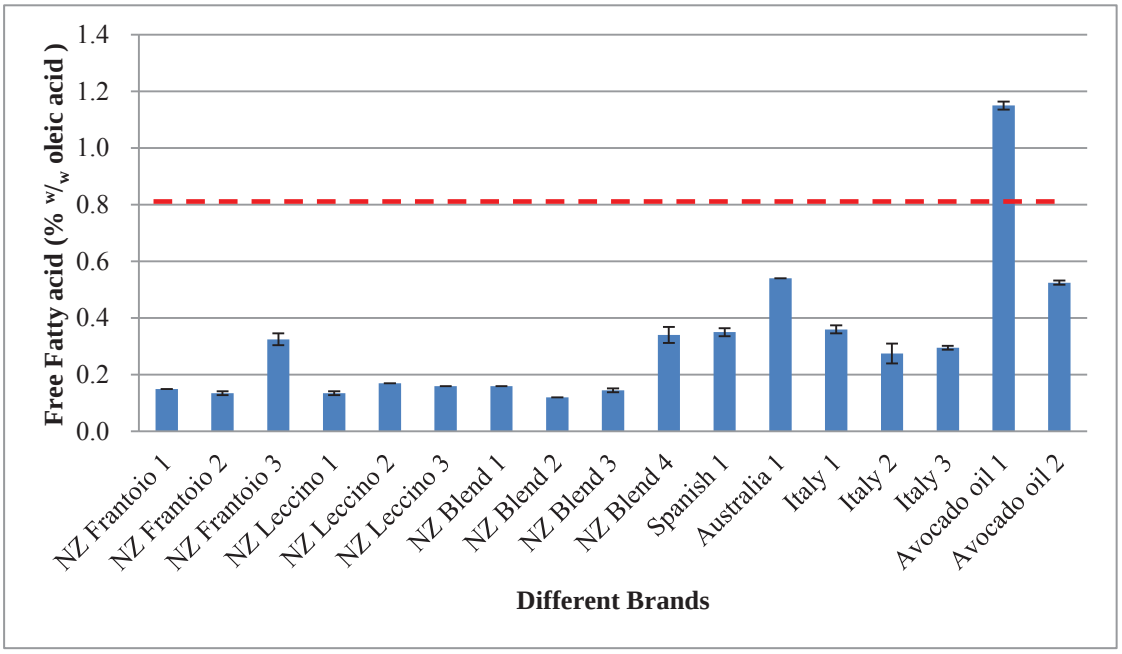


Figure 29. Comparison of free fatty acid for commercial oils. (mean ± standard deviation, n=2)

6.1.3 Total phenolics for commercial oils

The total phenolics content for each commercial oil are presented in Figure 30. NZ 'Frantoio' 3, NZ Blend 4, Australia 1 and Italy 1 had high total phenolics content.

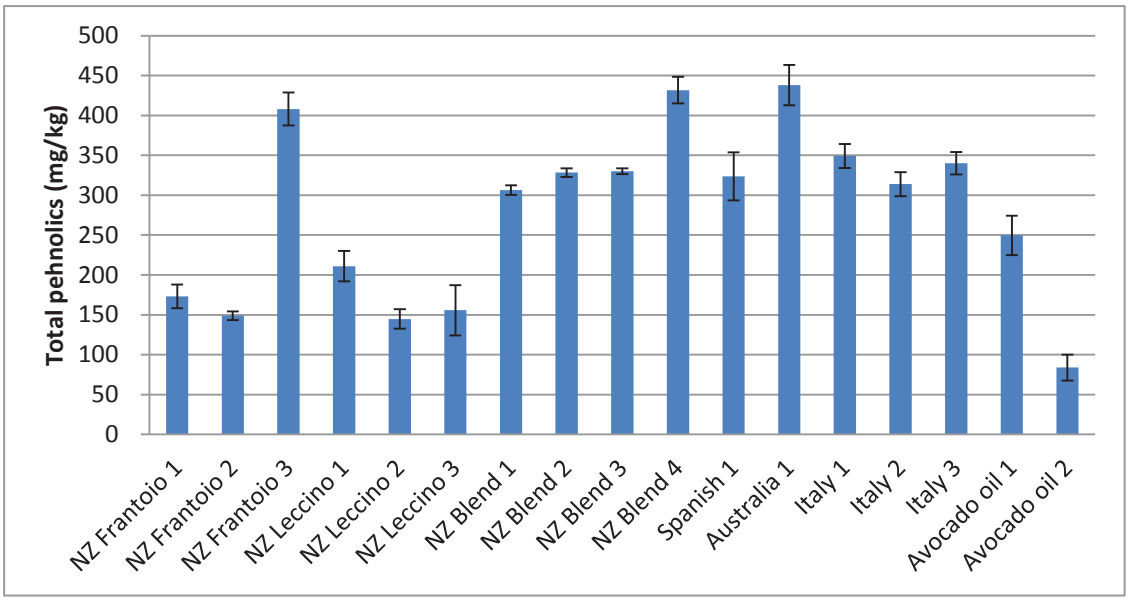


Figure 30. Comparison of total phenolic for commercial oils. (mean ± standard deviation, n=2)

6.1.4 Pyropheophytin % for commercial oils

As shown in Figure 31, the Spanish 1 olive oil had a very high percentage of pyropheophytin, which was over the limit (17%). It indicated that this oil did not fit into the EVOO category defined by the Australian Standards (Standards Australia, 2011). It also indicated that the oil had been stored for some time, this corresponded to the best before date on the bottle which indicated it only had 1 year shelf life left (Table 8). It was also notable that the Italy 1 oil had a significantly higher pyropheophytin percentage compared to olive oils produced in New Zealand and Australia and compared to the avocado oils, although they were all below the 17% limit. In a number of New Zealand olive oils, no pyropheophytins were detected (Figure 31). All the raw data of the cholorophyll a%, pheophytin a %, pheophytin a'% and pyropheophytin a % for commercial EVOOs and avocado oils are presented in Appendix 10.7. Chlorophyll a' was detected in avocado oil but not in any olive oils. The red dotted line in Figure 31 represents the limit set for pyropheophytin% in EVOO in the Standards Australia (2011).

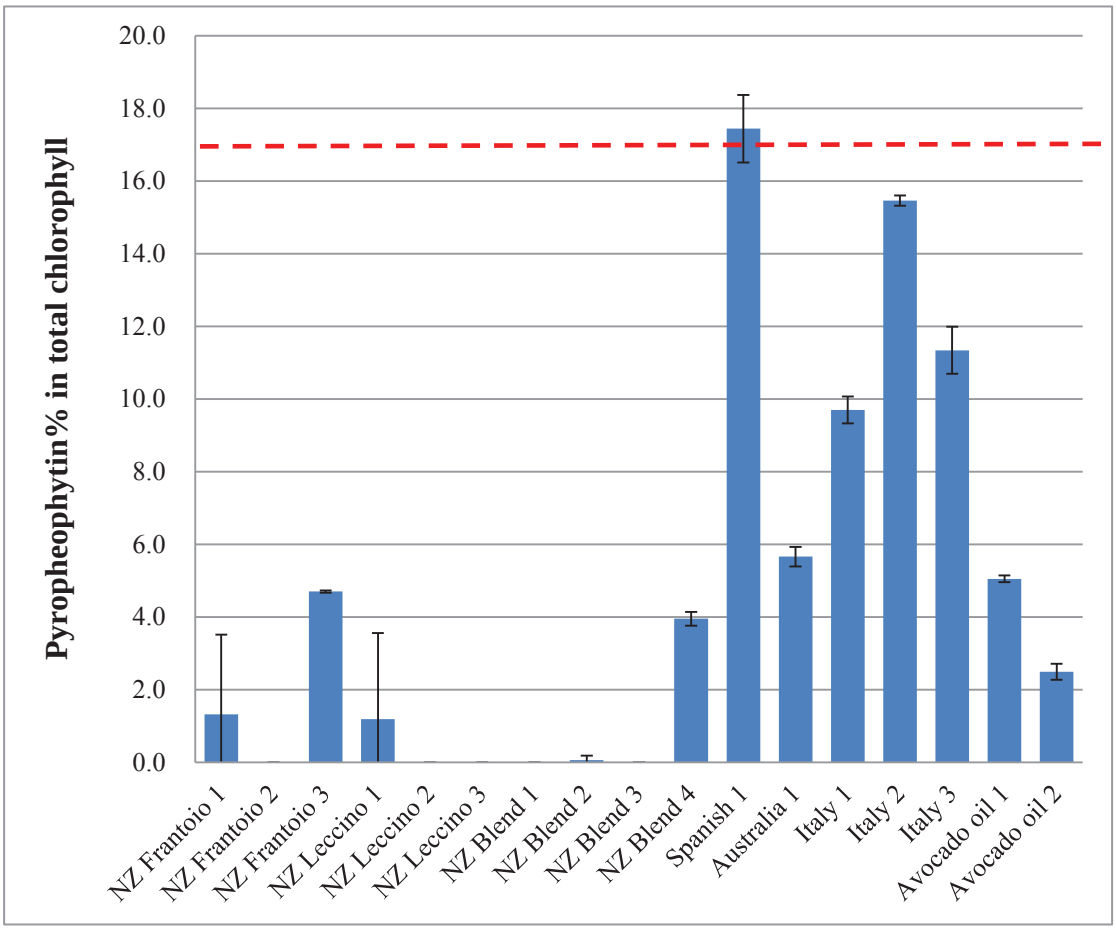


Figure 31. Comparison of pyropheophytin% for commercial oils (mean±standard deviation, n=2).

(The details for pyropheophytins of commercial oils were shown in Appendix 10.2)

6.1.5 Diacylglycerols % for commercial oils

The Australian Standard (Standards Australia) for 1,2-diacylglycerol% is that the EVOO must contain ≥ 35%. The total 1,2-diacylglycerol% comparison is shown in Figure 32. The Spanish 1 olive oil had a low percentage of 1,2-diacylglycerols and hence was below the recommended standard for DAGs (35%), this corresponds to the PPPs result which indicated the oil had been stored and it also had a Best Before date giving is a shelf life of only one year left. The olive oils Australia 1 and Italy 2 were

just above the limit at 35.64% and 36.87%, respectively. Conversely, the olive oils from New Zealand had high percentages of 1,2-diacylglycerol content, which indicated they were fresh. The red dotted line in Figure 32 represents for the lowest content of 1,2-DAGs/Total DAGs% present in EVOO in the Australian Standards (Standards Australia, 2011).

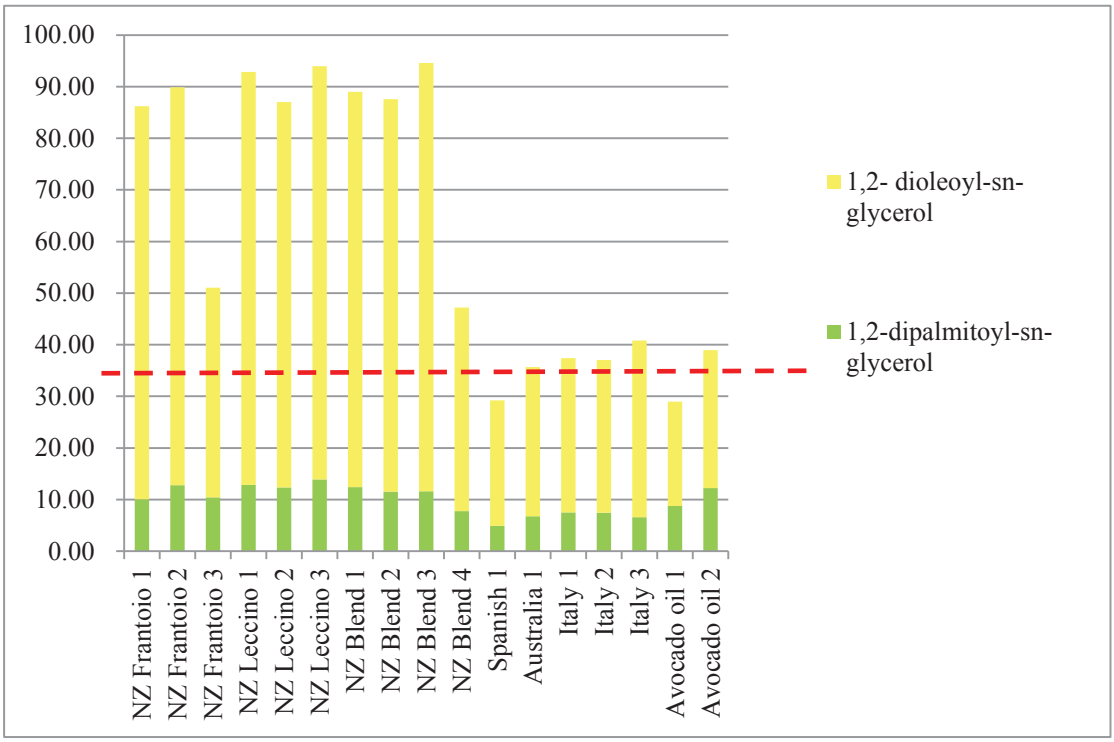


Figure 32. Comparison of total 1,2-diacylglycerol % for commercial oils.

6.2 Comparison between standards and the indices from New Zealand olive oils.

Compared to the overseas EVOOs, New Zealand EVOOs had more abundant quantities of 1,2-diacylglycerols and lower pyropheophytin%. The olive oil imported from Spain in the overseas commercial EVOO group had lower total 1,2-diacylglycerol% and high pyropheophytin%, which exceeded the limits set by Standards Australia (2011). Moreover, the Australia 1 oil just meet the Australian

Standard with the 1,2-diacylglycerols, although it had acceptable pyropheophytin%. While Italy 3 EVOO had more 1,2-diacylglycerols% in the overseas group. For the Avocado oil 1 and 2, the pyropheophytin% were 5.05% and 2.49% respectively and 1,2-diacylglycerols% were 28.93% and 38.97% respectively. All the tested oils were within the Australian Standard (Standards Australia, 2011) and International Olive Council (IOC) (2010a) standard's limits for PV and free fatty acid content. Most of the overseas' EVOO had over 300 mg/kg for total phenolics content, especially for the oil Australia 1 which had over 400 mg/kg, which means it contained abundant antioxidants in the oil.

The Standards Australia (2011) includes pyropheophytin% and 1,2-diacylglycerols% for quality to indicate stability of the olive oil. These tests would be helpful in determining quality of oils. The chemistry analysis is one part of EVOO determination in this commercial comparison and the other part of sensory analysis should also be also included, but if a correlation between sensory and pyropheophytin% and 1,2-diacylglycerols% can be found, testing for pyropheophytin% and 1,2-diacylglycerols% would be easier than sensory analysis.

It is interesting that one of the EVOOs from Europe failed the Standards Australia (2011). However, it met the IOC standard (2010). Also, Mailer, et al. (2010) reported that the results for olive oil from the major cultivars grown in Australia do not meet some of the limits set by international standards for some parameters in some cases for sterol composition. It implies that the olive oil standards need to be set by local government. Also concluded was that the EVOOs from European origin may have less shelf life than the New Zealand oils. It is notable that the New Zealand oil had good results for the pyropheophytin% and 1,2-diacylglycerols% which is only included in the Australian standards (Standards Australia, 2011).

6.3 Quality indices of commercial avocado oils

The recommended international quality standards for extra virgin avocado oil are free fatty acid (% as oleic acid) ≤ 0.5% and peroxide value ≤ 4 meqO_2/kg (Woolf et al., 2009). Avocado oil 1 had peroxide and free fatty acids above the recommended standards in Woolf et al (2009). This avocado oil had low level of pyropheophytin % but also low levels of 1,2-diacylglycerols%. The high %free fatty acids and low 1,2-diacylglycerols% indicate the oil may have been exposed to higher temperatures during extraction (Kodali et al., 1990; Fureby et al., 1996). Low level of pyropheophytin% in this oil implies it has not been stored for long. Avocado oil 2, had low PV, % free fatty acid, pyropheophytin% and 1,2-diacylglycerols%. The low level of 1,2-diacylglycerols% could not be explained. Thus, PPPs and DAGs test could be used for avocado oil to show the oil's age but the limits may be different from EVOO and required more investigation.

Chapter Seven: Overall Discussion

The first aim of this study was to describe the quality changes of two varieties ('Frantoio' and 'Leccino') of EVOO during storage in terms of peroxide value, K values, total phenolics, bitterness, pyropheophytin% and 1,2-diacylglycerols%. The second aim was to predict the shelf life of EVOO by kinetics using data on the peroxide value, pyropheophytin% and 1,2-diacylglycerols% changes. The final aim was to compare different commercial oils for EVOO (cultivars: 'Frantoio', 'Leccino' and Blends) and avocado oils from different countries (New Zealand, Australia, Italy and Spain) by comparing the free fatty acid content, peroxide value, total phenolics, pyropheophytin% and 1,2-diacylglycerols%.

'Frantoio' and 'Leccino' are two of the main Italian cultivars (Aguilera et al., 2005). Ranalli et al. (1998) reported that the quality of olive oil is chiefly affected by the olive ripening stage. Therefore, it is important to ascertain the optimal phase of the drupe ripening process, which should correspond to the period of olive harvesting, to obtain oils of the best quality. Moreover, Aguilera et al. (2005) and Vossen (2007) also studied 'Frantoio' and 'Leccino', they found there were significant differences between the oils from both cultivars when grown in the different environments. At higher altitude, the oils showed a greater content of oleic acid and higher stability, while the oils had higher tocopherol and linoleic acid contents at lower altitude. For the phenolic compounds, the environment influenced each cultivar in different ways. Sensorial characteristics showed significant differences between the oils from each cultivar and location. 'Frantoio' has a high and constant productivity and adaptation capacity to different agronomical conditions; although cold-sensitive; its organoleptic characteristics and oxidative stability are much appreciated. 'Leccino' is cold tolerant with an early start and high yield, a low resistance to fruit drop has been described, as well as tolerance against different plant diseases (Aguilera et al., 2005; Vossen, 2007).

Although there was much difference on the olive quality when they were harvested, there were slight differences in the quality stability between the two cultivars found in this study. Generally, the predicted shelf life for 'Frantoio' is slightly longer than 'Leccino'.

Lipolysis and oxidation are the processes leading to the deterioration of olive oil. Lipolysis usually starts when the oil is still in the fruit harvest, while the oxidation begins at the processing stage and proceeds during storage. Thus, although extra virgin olive oil is considered to be stable oil due to the presence of natural antioxidants, it is also susceptible to oxidation (Cosio et al., 2007; Méndez & Falqué, 2007). In this research, the degradation of EVOO from lipolysis was determined by monitoring free fatty acids, and oxidation was monitored with peroxide value. The two new tests monitor the breakdown of chlorophyll and 1,2-diacylglycerols both indicate that the oil has been stored for extended periods or has been exposed to temperature abuse. The overall changes for the storage of EVOO ('Frantoio' and 'Leccino') are distinct increases in PV and pyropheophytin%, and a significant loss of 1,2-diacylglycerols% at 30 and 40 °C. There was a slight increase in PV and pyropheophytin%, and a small loss of 1,2-diacylglycerols% at 7 and 20 °C. Total phenolics decreased significantly during the first three months and kept stable as the storage period continued. However, bitterness did not change as dramatically as phenolics and K values remained within the IOC and Australian Standard limits. In autoxidation olive oil stability has been correlated with the phenol content (Psomiadou & Tsimidou, 2002). However, the overall data showed that even though phenolics are antioxidants that can react as free radical scavengers in the oil and their concentrations are associated with stability of the oil (Ninfali et al., 2002), the EVOO still aged on storage.

Lipid oxidation takes place through a set of autocatalytic reactions that produce a high number of new compounds. Hydroperoxides, the primary oxidation compounds, decompose giving rise to a variety of secondary oxidation products. The analysis of primary oxidation compounds is always carried out either by the peroxide value or K values or sensory analysis (Marmesat et al., 2009). In general, the oil has lower PV and lower K values and then the better quality of the oil. However, PV decreases as secondary oxidation products appear. Oils with significant levels of peroxides may still be odourless if secondary oxidation has not begun. If oxidation occurs more advanced, the PV may be relatively low. However, the oil will be obviously rancid (Gutierrez & Fernandez, 2002; Mancebo - Campos et al., 2008). Thus, the PV test is useful for predicting shelf life when the storage period was short or used together with other tests, such as a Rancimat, during a shelf life study (Hamilton, 1994). The predicted shelf life as PV trends in this study was more than 2 years for 'Leccino' and more than 3 years for 'Frantoio' at 20 °C, respectively. This predicted shelf life was predicted to be shorter when using pyropheophytin% results but longer when predicted with the 1,2-diacylglycerols% results. There were no published data found relating the predicted shelf life of EVOO using these two tests.

There is a limit on pyropheophytin% which is 17% in the Standards Australia (2011). This chlorophyll degradation products (pyropheophytin%) provide information about the storage conditions. It can be detected sensitively by HPLC in this storage trial and had clear trends of the results at four temperatures. This revealed pyropheophytin% is an ideal parameter for tracing the deterioration of the quality of EVOO, which is in agreement with the report by Aparicio-Ruiz et al. (2012).

Data for the 1,2-diacylglycerols% determined by GC gave an indication of storage stability at the storage temperatures. The 1,2-diacylglycerols% decreased as the storage period was prolonged and the rate of decrease increased with increasing temperature, in agreement with the results by Pérez-Camino et al. (2001). However,

the oils still met the Australia Standards (2011) at the end of the storage trial. In this research, only 1,2-dilinolein; 1,3-dilinolein; 1,2-diolein and 1,3-diolein can be found following the ISO 29822: 2009 (E) standard methods.

To monitor the quality of EVOO, all the parameters for PV, pyropheophytin% and 1,2-diacylglycerols% should be included. Also, volatile aromatic compounds and also some nonvolatile compounds, strongly affecting sensory receptors, can decisively influence the food acceptability, direct the preference of the consumer and determine the quality of oil to a great extent (Angerosa, 2002). However, for the sensory evaluation, there is no cheap way to detect the volatile compounds now, although they are mainly responsible for the pleasant flavours and changing into off-flavours during storage (Cosio et al., 2007).

All oils used in the commercial EVOO comparison gave different results on free fatty acid, peroxide value, total phenolics, pyropheophytin% and 1,2-diacylglycerols%. It is noteworthy that the comparison among the commercial EVOOs produced in different countries showing that one brand of EVOO produced in Spain (Spanish 1) did not meet the EVOO standards for Standards Australia (2011), although Spain is one of the major countries that produces olive oil in Europe (Bonanome, et al., 2005; Guzmán & Alonso, 2008; López-Miranda et al., 2010). This indicates the EVOO from Spain had been stored for a long time. The other imported oils were still within the various standards for extra virgin status. The EVOOs produced in New Zealand showed good quality in the storage trial as well as in the commercial comparison study, especially on the pyropheophytin% and1,2-diacylglycerols% results.

This is the first time the pyropheophytins and 1,2-diacylglycerols have been tested in NZ oils. The pyropheophytins and 1,2-diacylglycerols tests are complicated, which require more technical skills. The results obtained from these tests were found to be reproducible with small variability between replicates. All imported EVOOs have

higher level of PPPs and lower DAGs than New Zealand EVOOs, which indicates the imported oils are older. However, their peroxide value and free fatty acid are still within specification of the different standards. Therefore, the chemical analysis should be used in combination with other tests such as PPPs, DAGs and sensory evaluation. The aged oil cannot be detected by a single analysis.

Chapter Eight: Conclusions and Recommendations

8.1 Conclusions

In this study, it was found that

1. The quality of olive oil cannot be predicted with just one test but should include chemical tests of peroxide value, pyropheophytin and 1,2-diacylglycerols plus sensory evaluation.
2. The pyropheophytin and 1,2-diacylglycerols tests using HPLC and GC, respectively, were successfully used to test olive oil quality.
3. Good quality of EVOO have a high content of phenolic compounds (approximately 1000 mg/kg) and a high content of 1,2- DAGs (up to 90% of total DAGs).
4. The quality of EVOO is affected by temperature, especially if the temperature is above 20 °C.
5. New Zealand EVOO can be kept for at least one and half years at 20 °C and more than six years at 10 °C based on the combination of PV, PPPs and DAGs chemical analysis.
6. EVOO produced in New Zealand for the olive oil awards had the best quality based on the FFA, PV, total phenolics, PPPs and DAGs tests after comparison with different brands and producing countries of EVOO.
7. Although PV and free fatty acid concentrations were within the standard limit set by the Australian Standards, the quality of olive oil still should still be analysed to confirm whether it is can be classified as EVOO by testing for PPPs and DAGs analysis.
8. The stability of phenolic compounds is affected by storage time, rather than temperature.

9. Pyropheophytin and 1,2-diacylglycerols tests can be used to monitor deterioration in oils.

8.2 Recommendations

The present study revealed that the any single chemistry analysis is not enough for evaluating the extra virgin classification of olive oil. The combined analysis with sensory in parallel is the requirement.

Future studies involving sensory evaluation could be used to distinguish the quality between two varieties (‘Frantoio’ and ‘Leccino’) of EVOO.

Chapter Nine: References

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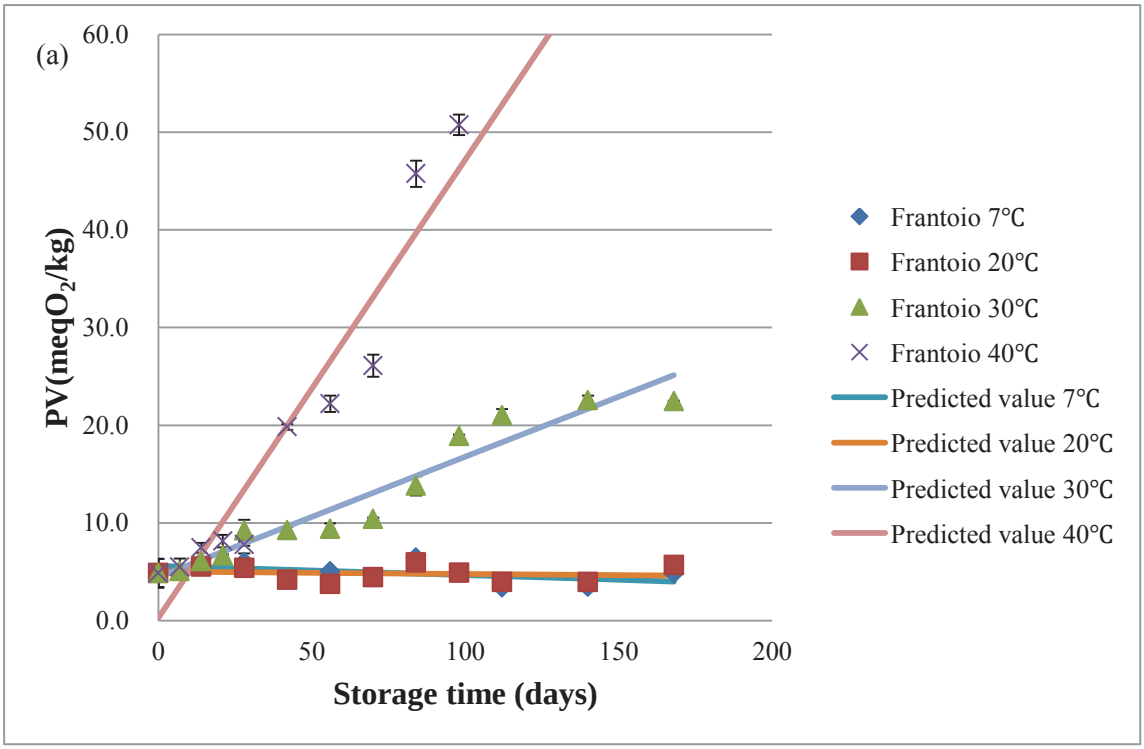
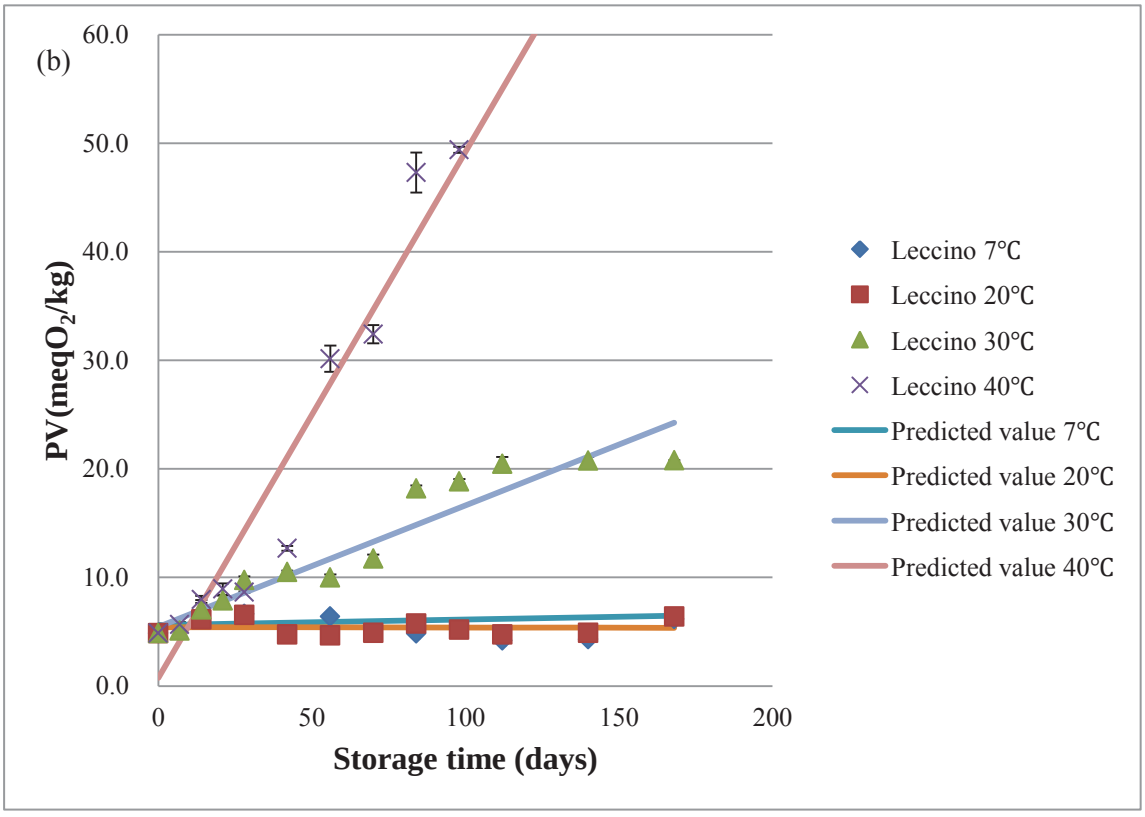
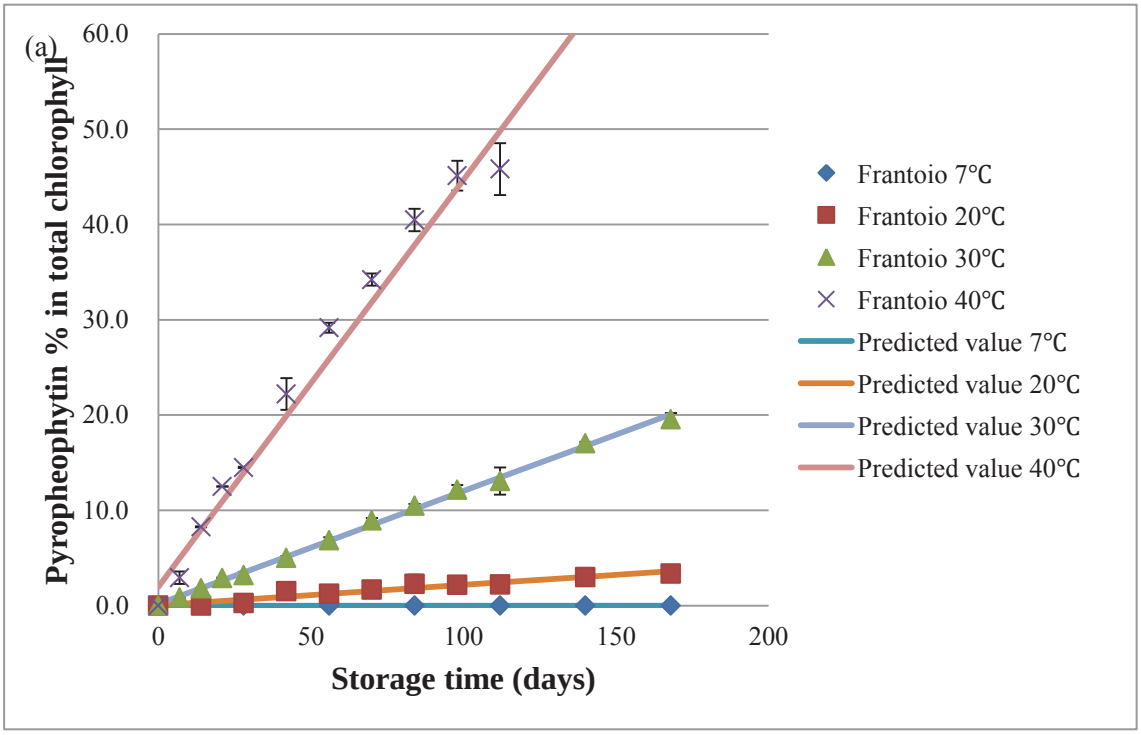
Chapter Ten: Appendices

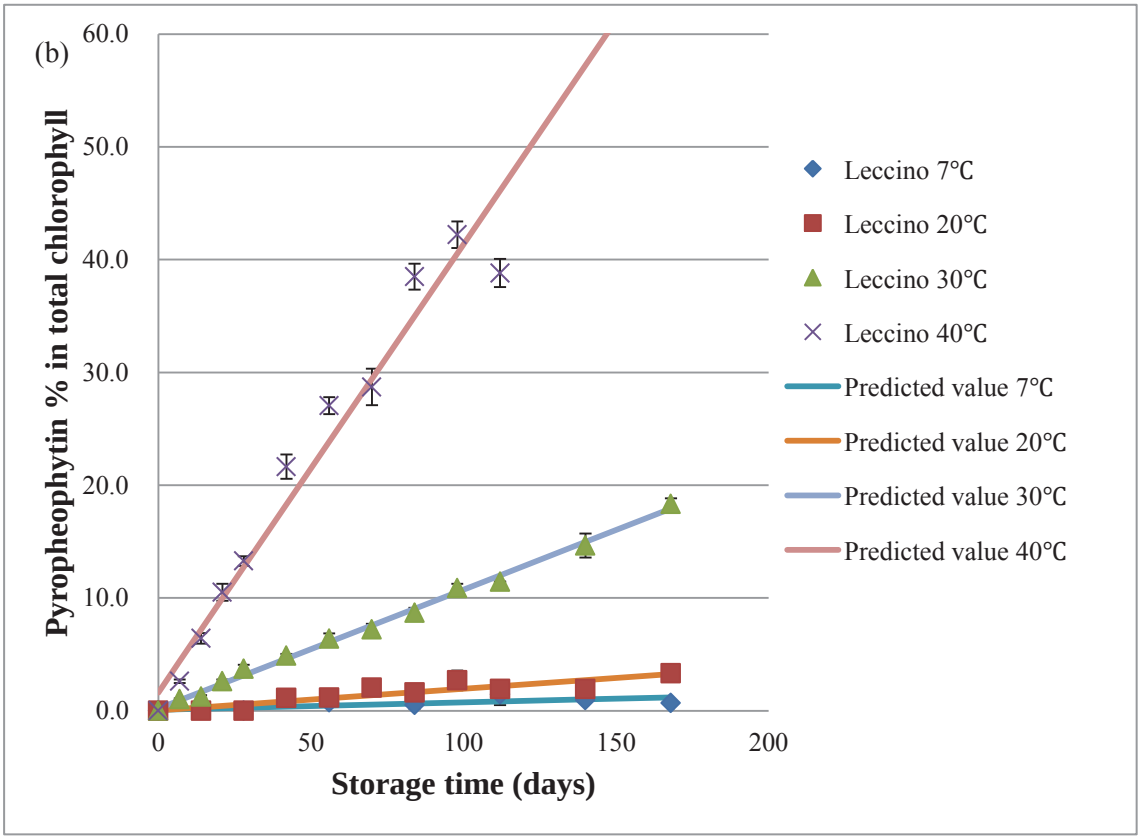
Appendix 10.1 The predicted trends of four different temperatures of EVOO based on the zero order results of PV. (a): 'Frantoio'; (b): 'Leccino'.



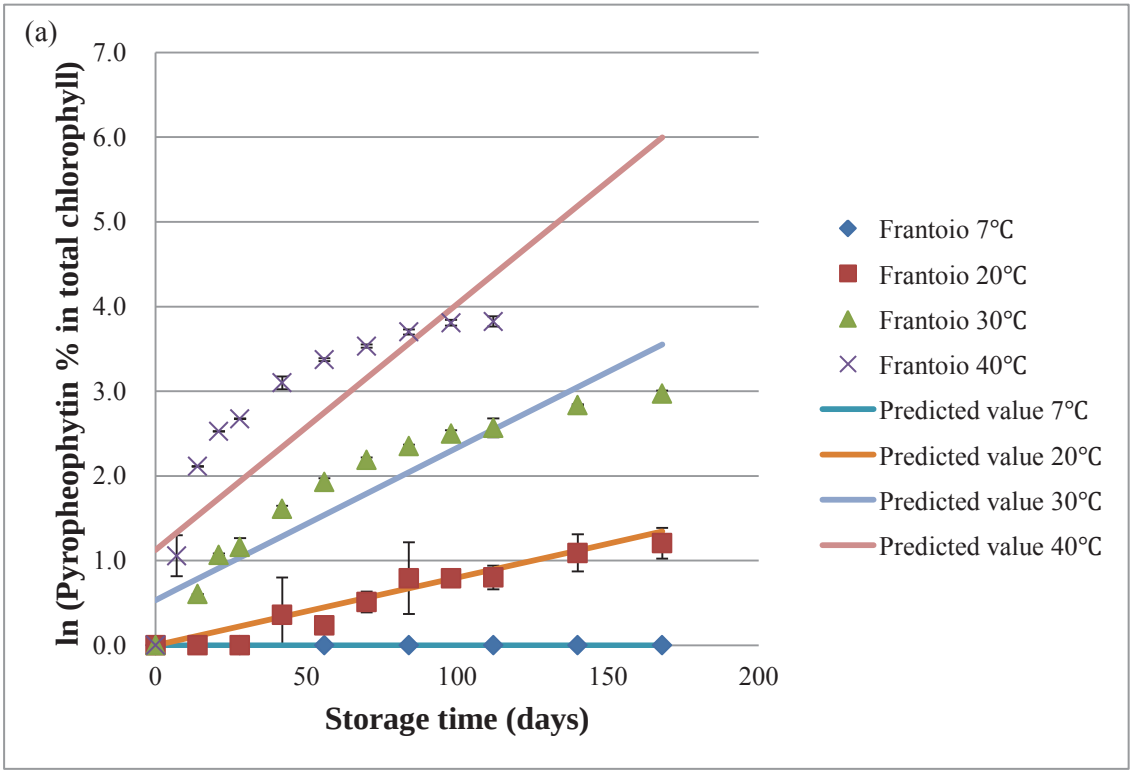


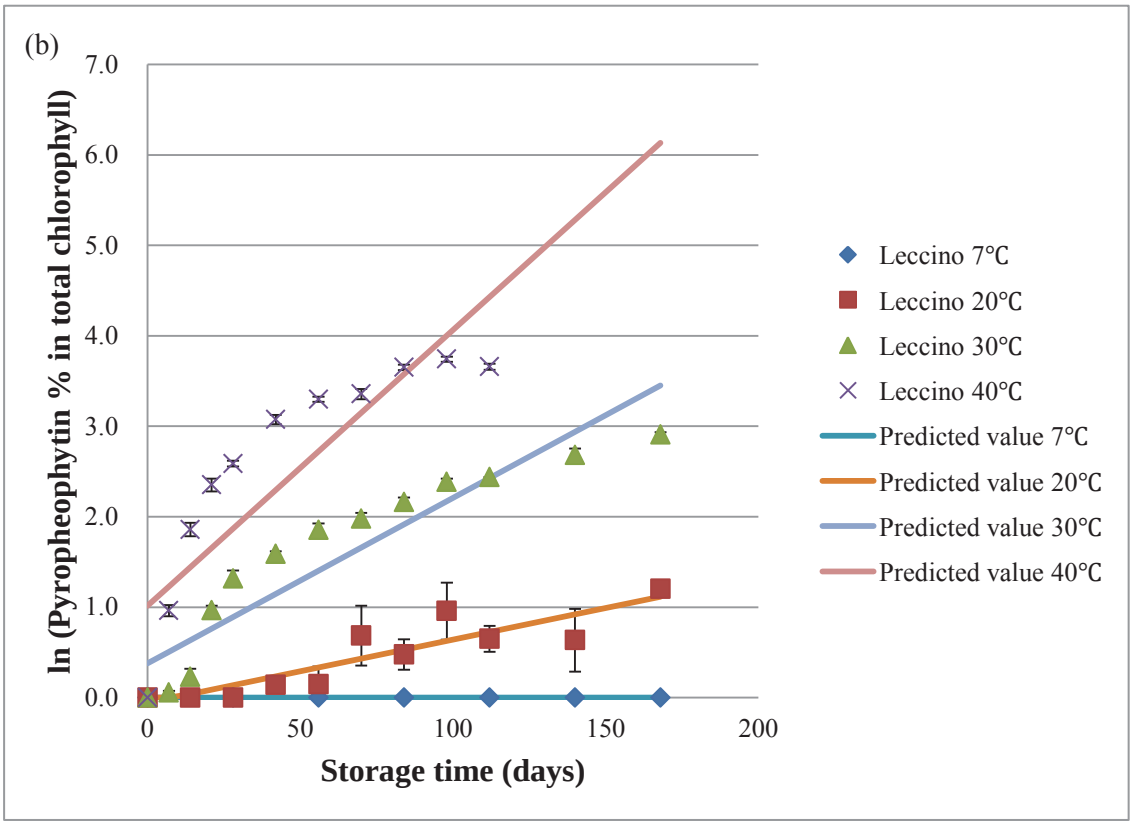
Appendix 10.2 The predicted trends of four different temperatures of EVOO based on the zero order results of Pyropheophytin. (a): 'Frantoio'; (b): 'Leccino'.



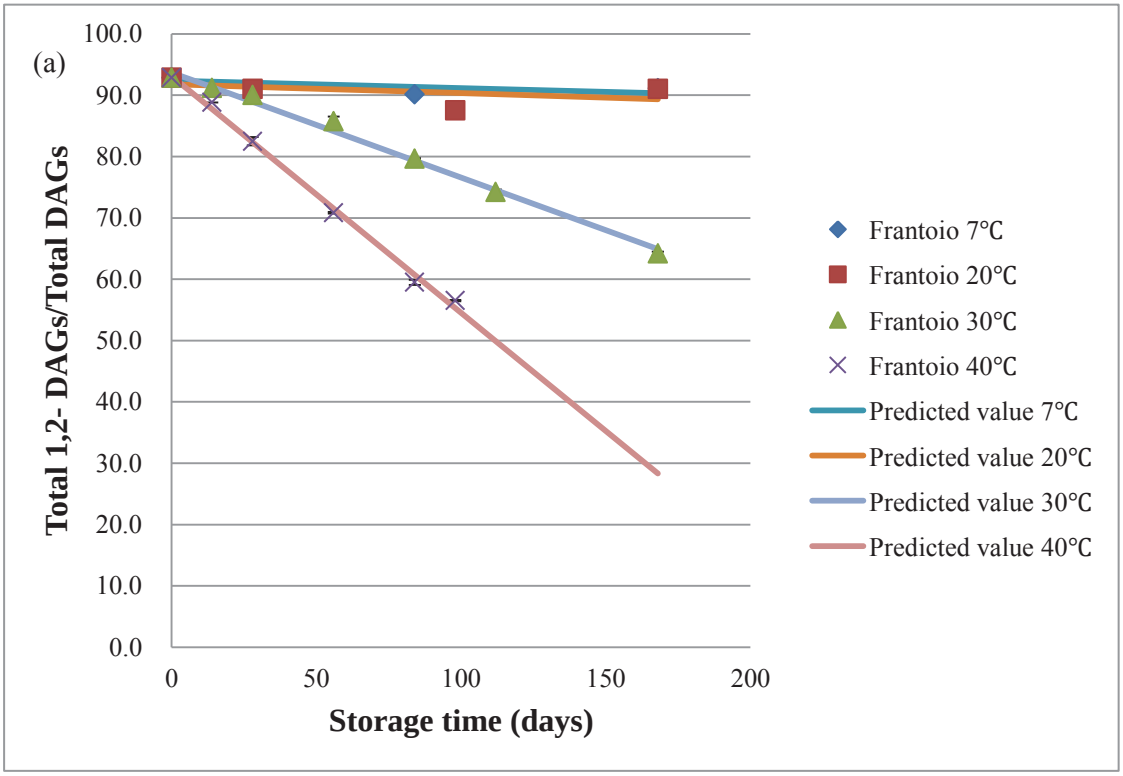


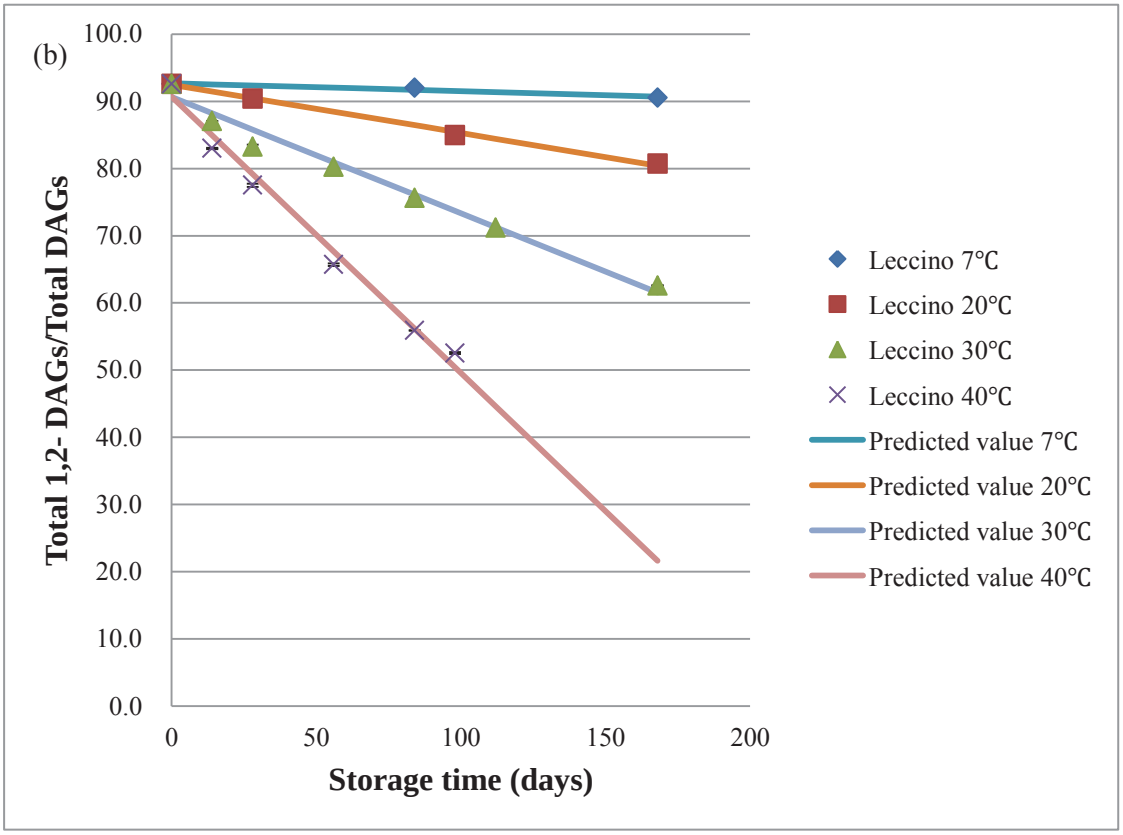
Appendix 10.3 The predicted trends of four different temperatures of EVOO based on the first order results of Pyropheophytin. (a): 'Frantoio'; (b): 'Leccino'.



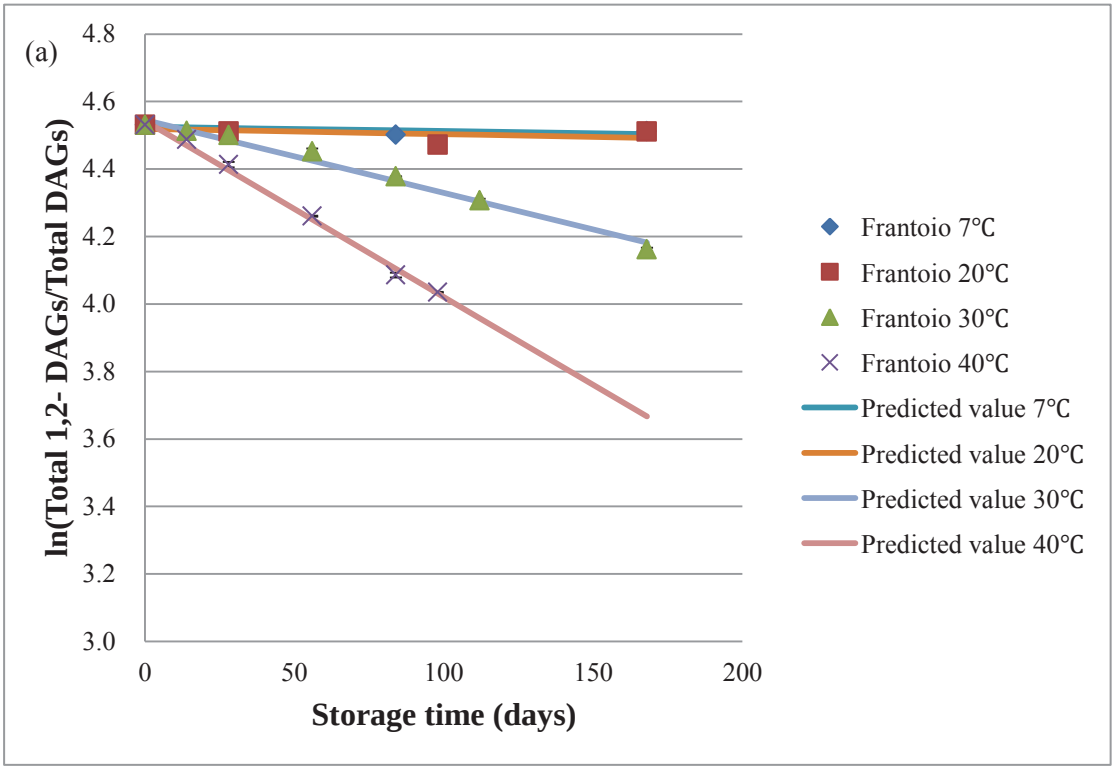


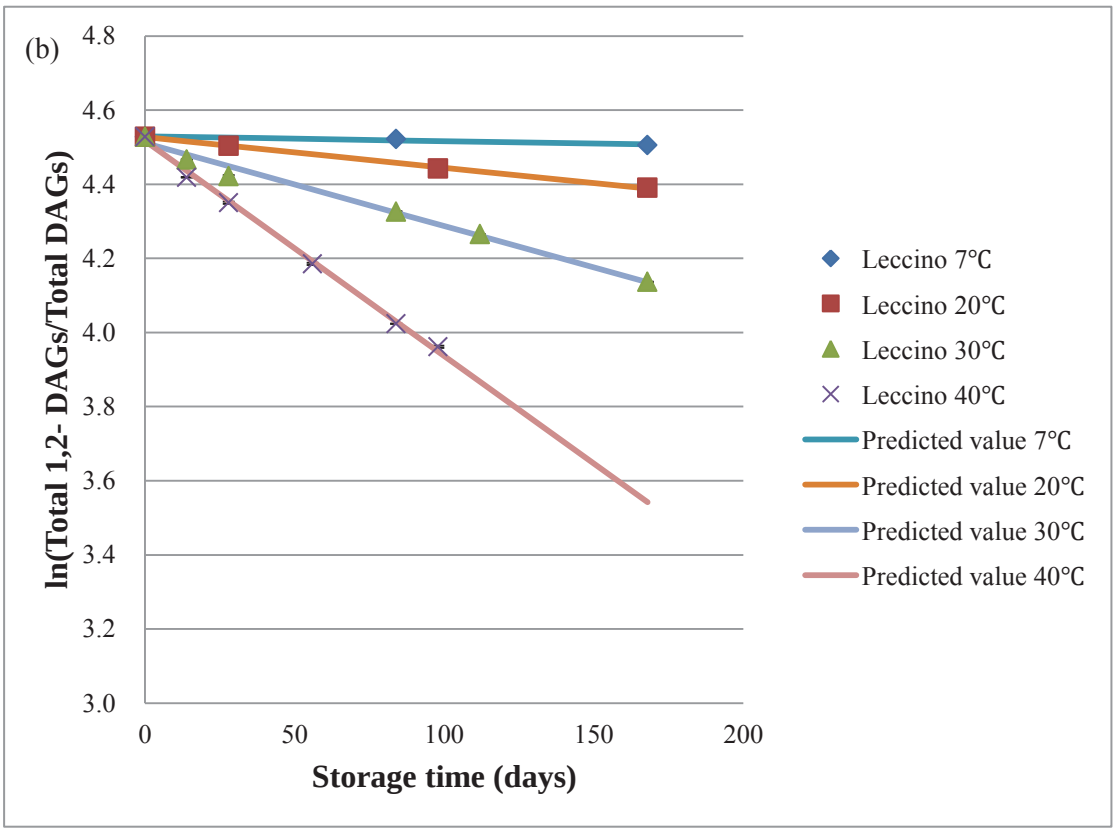
Appendix 10.4 The predicted trends of four different temperatures of EVOO based on the zero order results of DAGs. (a): 'Frantoio'; (b): 'Leccino'.





Appendix 10.5 The predicted trends of four different temperatures of EVOO based on the first order results of DAGs. (a): 'Frantoio'; (b): 'Leccino'.





Appendix 10.6

An example for shelf life estimation basing on zero order of peroxide value ('Frantoio', 10 ℃)

$$\ln k = -\frac{Ea}{R} \times \frac{1}{T} + \ln A$$

$$\ln k = -16465 \times \frac{1}{T} + 51.723$$

For 10 ℃ = 283 K

$$\ln k = -16465 \times \frac{1}{283} + 51.723$$

$$\ln k = -6.457$$

$$k = 1.569 \times 10^{-3}$$

$$PV_{\max} - PV_{zero} = kt$$

$$\frac{20 - 4.85}{1.569 \times 10^{-3}} = t$$

$$t = 9655 days = 26.5 years$$

An example for shelf life estimation basing on first order of DAGs (‘Frantoio’, 10 ℃)

$$\ln k = -\frac{Ea}{R} \times \frac{1}{T} + \ln A$$

$$\ln k = -10617 \times \frac{1}{T} + 28.496$$

For 10℃ = 283 K

$$\ln k = -10617 \times \frac{1}{283} + 28.496$$

$$\ln k = -9.020$$

$$k = 1.210 \times 10^{-4}$$

$$\ln PPP_{\max} - \ln PPP_{zero} = kt$$

$$\frac{\ln 92.87 - \ln 35}{1.210 \times 10^{-4}} = t$$

$$t = 8065 days = 22.1 years$$

Appendix 10.7

Brands	Cholorophyll a % in total Chlorophyll	Pheophytin a % in total chlorophyll	Pheophytin a'% in total chlorophyll	Pyropheophytin% in total chlorophyll
NZ 'Frantoio' 1	0.71 ± 1.06	82.98 ± 4.09	15.00 ± 1.92	1.32 ± 2.19
NZ 'Frantoio' 2	0	89.53 ± 1.48	10.47 ± 1.48	0
NZ 'Frantoio' 3	0.34 ± 0.41	80.82 ± 1.15	12.57 ± 1.02	4.70 ± 0.03
NZ 'Leccino' 1	1.04 ± 0.09	84.43 ± 0.21	14.52 ± 0.13	1.19 ± 2.37
NZ 'Leccino' 2	1.92 ± 0.07	85.26 ± 0.08	12.82 ± 0.15	0
NZ 'Leccino' 3	0	85.61 ± 0.51	14.39 ± 0.51	0
NZ Blend 1	0.39 ± 0.07	85.12 ± 0.21	14.49 ± 0.15	0
NZ Blend 2	0.38 ± 0.44	84.93 ± 0.44	14.63 ± 0.09	0.06 ± 0.12
NZ Blend 3	2.09 ± 0.04	84.38 ± 0.09	13.53 ± 0.08	0
NZ Blend 4	0.28 ± 0.4	82.88 ± 0.88	12.88 ± 0.68	3.95 ± 0.19

Brands	Cholorophyll a % in total Chlorophyll	Pheophytin a % in total chlorophyll	Pheophytin a'% in total chlorophyll	Pyropheophytin% in total chlorophyll
Spanish 1	0	70.99 ± 0.64	11.57 ± 0.30	17.44 ± 0.93
Australia 1	0.16 ± 0.32	81.07 ± 1.93	12.52 ± 2.10	5.66 ± 0.27
Italy 1	0	77.11 ± 0.14	13.19 ± 0.51	9.7 ± 0.37
Italy 2	0	72.78 ± 0.49	11.76 ± 0.62	15.46 ± 0.14
Italy 3	0	75.71 ± 1.29	12.95 ± 0.64	11.34 ± 0.65
Brands	Cholorophyll a % in total Chlorophyll	Cholorophyll a' % in total Chlorophyll	Pheophytin a % in total chlorophyll	Pheophytin a'% in total chlorophyll
Avocado oil 1	3.15 ± 0.42	0.92 ± 0.40	75.99 ± 0.76	14.89 ± 0.17
Avocado oil 2	1.92 ± 0.25	0.52 ± 0.23	79.82 ± 0.39	15.25 ± 0.31