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Cold pressed avocado oil – impact of adding skin and seed during malaxing

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Abstract

The production of extra virgin cold-pressed avocado oil is based on the mechanical extraction of olive oil, involving grinding, malaxing and centrifugation, which mainly extracted oil from the mesocarp of avocado fruit. During processing a step in the process is included to eliminate skin and seed before extraction. The aim of this study was to determine the effect of incorporating avocado skin and seed during the cold-press extraction process on oil yield, oil composition and oil storage stability. Laboratory-scale extraction equipment was used to extract oil from 'Hass' avocados, harvested in July 2022 (late season fruits from the previous season) and in August 2022 (early season fruits for the new season). Three different tissue combinations in the malaxers were investigated: flesh with 10% of the skin tissue, flesh with 100% skin, flesh with 100% skin and seed (whole fruit). Each oil was immediately transferred into amber bottles, nitrogen flushed the headspace and then stored at 20°C, 30°C and 40°C for the storage trial. Oil samples were also stored at -20°C to be used for analysis of composition. The oil yield (kg oil/ (kg of wet tissue in malaxer)) from malaxing whole fruit was considerably less than from malaxing flesh with 10% skin; 65% and 35% less for early and late season, respectively. Adding skin and seed did not significantly change the free fatty acid and peroxide value in oil, although the UV absorbance values (K_{232} and K_{270}) were higher in whole fruit oil. While there were no significant differences in fatty acid and sterol profiles, the whole fruit oil contained significantly higher concentrations of phenolics, sterols and pigments (carotenoids and chlorophylls) ($p < 0.05$). During storage, oils at higher storage temperatures exhibited a more rapid increase in peroxide value (PV) and K values, along with a more rapid decline in total phenolics and total chlorophyll. Informal sensory trials suggested that the bitterness of the oil increased with storage time, though sensory was not evaluated. It can be concluded that the addition of skin and seed led to a reduction in oil yield due to the lower proportion of flesh mass in malaxer, however more liposoluble compounds dissolved in the oil. Although the Rancimat test indicated improved oxidative stability with the addition of skin and seed, the whole fruit oil demonstrated a higher oxidation rate during storage, coupled with increased sensitivity to temperature.

Key words: extra virgin avocado oil, cold-pressed, skin, seed, composition, stability

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Chapter 1: Introduction

Avocado oil is becoming increasingly popular due to its unique flavour and high nutritional value (Tan, 2019). Extra virgin avocado oil has gained more interest for its higher concentrations of antioxidants and pigments, as well as its simple green mechanical production (Cervantes-Paz & Yahia, 2021; Green & Wang, 2022b; King-Loeza *et al.*, 2023; Wong *et al.*, 2010; Woolf *et al.*, 2009). The cold-pressed extraction process extracts oil primarily from the mesocarp of avocado fruit, leading to a significant amount of avocado skin and seed being generated as by-products (Araújo *et al.*, 2018; Cárdenas-Castro *et al.*, 2023). Currently, most of the literature on avocado oil focuses on optimizing extraction conditions, with limited research on the impact of skin and seed on extra virgin avocado oil (Costagli & Betti, 2015; Martínez-Padilla *et al.*, 2018; Pérez-Saucedo *et al.*, 2021). Green and Wang (2022b) have recently studied the quality impact of oil extraction from avocado mesocarp and from the whole fruit on extra virgin avocado oil and found that using whole fruits can also meet the standards of extra virgin grade. The skin is known to contain more pigments and the seed contains high content of phenolics and sterols (Araújo *et al.*, 2018; Ashton, 2005; Ashton *et al.*, 2006; Cárdenas-Castro *et al.*, 2023; Ejiofor *et al.*, 2018; Wong *et al.*, 2011). These inclusions provide the possibility of obtaining higher quality avocado oil with additional functional components by incorporating the skin and seed during oil extraction. Avocado oil as with other lipids is very prone to oxidation. Due to the spontaneous nature of oil oxidation, deterioration happens during the storage of oil (Boskou, 2006). Some crucial quality parameters are often utilized to assess the oil quality, such as free fatty acid (FFA) and peroxide value (PV) (Brzezińska *et al.*, 2020). Although the standards for avocado oil are not yet established, Codex Alimentarius Commission has proposed a standard as a reference (Codex Alimentarius Commission, 2021).

This study utilized avocados harvested in both early and late season, where three different combinations of flesh, skin and seed during malaxing were evaluated: flesh with 10% of the skin tissue, flesh with all skin, flesh with all skin and seed. The first objective of this study was to determine the effect of different amounts of flesh, skin and seed added during malaxing on the composition of the oil extracted using cold-pressed oil extraction. The second objective of this study was to determine the effect of the different components present in the oil after extraction, on the storage stability of cold-pressed avocado oil. Various techniques were used to quantify the composition based on published literature (Caligiani *et al.*, 2010b; López-Cobo *et al.*, 2016; Mateos & García-Mesa, 2006). The quality of the oil was assessed using standard methods from AOCS (2017) including, FFA, PV and coefficients of specific extinction (International Olive Council, 2019a), as well as total phenolic content (TPC) (Singleton *et al.*, 1999; Vinson *et al.*, 2005). The results from this study will provide information on oil yield, oil composition and oil stability produced with the addition of skin and seed from fruit collected twice during the avocado season, which could provide a recommendation for the utilization of avocado skin and seed in the production of extra virgin avocado oil, thereby enhancing the oil quality and increase storage stability.

Chapter 2: Literature review

2.1 Overview of avocado fruits

Avocado (*Persea americana Mill.*) is a tropical/subtropical fruit with unique flavour and creamy texture (Cervantes-Paz & Yahia, 2021). Due to its high nutritional value and high oil content, the second grade fruit that do not meet local-market or export quality standards are usually utilized for extracting oil for the food industry (Woolf *et al.*, 2009).

2.2 Avocado fruit production

Avocado belongs to the *Lauraceae* family. Avocados are perceived to provide health benefits and also culinary versatility (Schaffer *et al.*, 2013). They are classified into eight distinct geographical eco-types, commonly known as horticultural races (Heiser, 1979). Three of them comprise the main commercial avocado crop, which are the the Mexican (*P. americana var. drymifolia*), the Guatemalan (*P. nubigena var. guatemalensis and P. nubigena var. nubigena*) and West Indian (*P. americana var. americana*) varieties (Heiser, 1979). The current commercial avocado varieties are hybrids derived from these horticultural races. The 'Hass' variety is classified as a hybrid belonging to the Guatemalan-Mexican group (Chanderbali *et al.*, 2008). 'Hass' is extensively cultivated in Mexico and California, and is one of the primary commercially grown avocado varieties (Lu *et al.*, 2009). Its fruits are a pear to ovoid shape, with a tough, leathery skin that turns dark brown or black when fully ripe (Woolf *et al.*, 2009).

FAOSTAT (2020) reports that global avocado production increased significantly over the last decade. From 2011 to 2020, avocado production has doubled from 4.07 million tons to approximately 8.06 million tons, representing a 50% growth. The adaptability of the avocado fruit to various tropical ecological zones has enabled it can be cultivated in more than 60 countries worldwide (Araújo *et al.*, 2018). In terms of individual country production, Mexico continues to be the leading producer and exporter of avocados, accounting for more than a third of the world's avocado production at 2,592,581 million tons (FAOSTAT, 2024). Colombia and Peru rank second and third, respectively, with production capacities of 1,090,664 and 866,457 tons (FAOSTAT, 2024).

The ‘Hass’ cultivar is dominant in lots of major avocado-growing countries, such as Chile, the United States and Mexico, as well as smaller producers like New Zealand, Australia and Spain. It accounts for more than 90% of total avocado production in these countries. The ‘Hass’ cultivar is primarily produced for fresh fruit export and then reject fruit can be used for extracting avocado oil and other processing purposes (Woolf *et al.*, 2009). Its popularity can be attributed to its postharvest characteristics, including good postharvest storage, high yielding variety, creamy texture and rich flavour when fully ripe and its tolerance to pests and diseases (Schaffer *et al.*, 2013). Additionally, the skin colour change from green to black serves as an indicator of fruit ripeness and helps conceal minor rind imperfections (Crane *et al.*, 2013).

From 1987 to 2020, avocado production in New Zealand witnessed a significant increase, growing from 1,000 tons to 40,000 tons (FAO, 2021; Requejo-Tapia, 1999). The potential for further growth in production remains considerable. The majority of first-grade avocados are exported overseas, whereas the local market takes the fresh fruit rejects, which primarily refers to defects in the cosmetic aspects of the fruit such as colour, shape, and size. The production of avocados in New Zealand varies every year, influenced by multiple factors, for example, season and weather conditions (Wong *et al.*, 2014).

a2.3 Maturity of avocado fruits

Fruit maturity can be defined as horticultural maturity or physiological maturity. Horticultural maturity is when to harvest the fruit at a stage of development that supports ripening for a desirable taste and quality, meeting consumer preferences. Physiological maturity in avocados refers to the stage of development at which the fruit has completed its growth, but can continue to undergo physiological changes after harvest (Lee *et al.*, 1983; Watada *et al.*, 1984). The maturity of avocado fruit plays a crucial role in determining the availability of avocado oil (Yang *et al.*, 2020).

Unlike many other fruits, avocados have the unique ability to remain on the tree for several months after reaching horticultural and physiological maturity. These fruits can ripen once they are removed from the tree (Schaffer *et al.*, 2013). During the period on the tree, ranging from 7 to 18 months, when the avocado fruit is mature, various important changes occur within the fruit (Woolf *et al.*, 2009). ‘Hass’ avocados over the season will change in both the average total oil content and dry matter content, as well as show a different yield for commercially cold-pressed oil (Figure 2.1) (Woolf *et al.*, 2009; Yang *et al.*, 2020). The difference in commercial oil yield between early-season and late-season fruit was not attributed to differences in ripeness, as both were extracted at the same ripeness stage. It was found that the oil yield of avocados was positively correlated with their maturity (Lee *et al.*, 1983; Yang *et al.*, 2020). The cold-pressed extraction of avocado oil from late-season fruit (April) resulted in higher yields compared to early-season fruit (October). For early season fruits, the commercial cold-pressed oil yield was approximately 13% (g oil/ 100g wet flesh), while the available content of total oil present in fruit was around 19% (g oil/ 100g wet flesh). As the season progressed, the total oil content of fruits went up to nearly 25%, hence improving the extraction yield. The underlying cause for the reduced efficiency of early-season compared to late-season fruit during cold-pressed oil extraction remains unclear. One possible explanation is that the strength of the cell walls become weaker as the season progresses, as shown by Yang (2019).

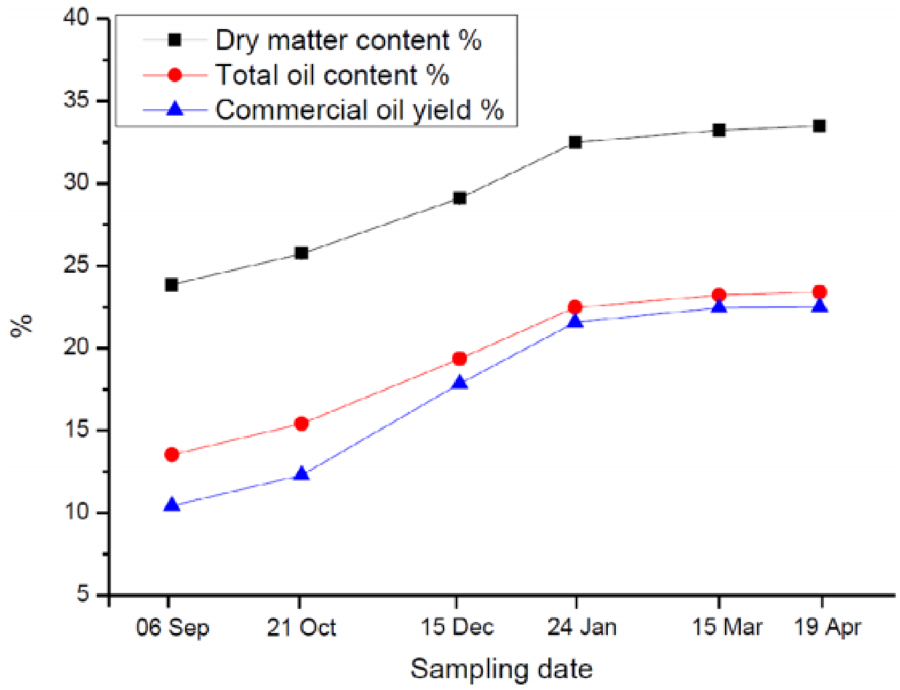

Figure 2.1: Typical variations of oil content (g oil/100g fresh flesh) and dry matter (g dry flesh/100g fresh flesh) in New Zealand 'Hass' avocados during 2016/2017 season and commercial harvest (Yang *et al.*, 2020).

2.3.1 Dry matter determination

The dry matter content in the flesh serves as the primary factor for assessing oil content and maturity in avocados. This measure is widely employed for the commercial harvesting of fresh fruit by the industry due to its simplicity, safety, and feasibility for packhouses and growers (Gamble *et al.*, 2010; Villa-Rodríguez *et al.*, 2011). The increase in dry matter content is used as in indicator for the total oil content and has been found to be correlated (Woolf *et al.*, 2009).

To determine the dry matter content in avocados, a common method involves extracting a sample of flesh tissue and subjecting it to drying until it reaches a constant weight. This process can be achieved using different techniques such as a dehydrator or an oven set at approximately 65°C. Alternatively, a domestic microwave can also be utilized for this purpose (Woolf *et al.*, 2009). Former research has demonstrated a strong positive correlation between the accumulation of oil content and dry matter in avocados (Lee *et al.*, 1983; Woolf *et al.*, 2009).

2.3.2 Ripening of avocado fruits

During the ripening process of 'Hass' avocados, the skin colour turns from green to black combined with the softening of the flesh (Table 2.1). However, determining the ripeness of fruits by only seeing the colour change in the skin of 'Hass' avocados is not always reliable. Currently, firmness is considered to be the most efficient and widely used method for determining the ripeness of avocados (White *et al.*, 2009).

Table 2.1: Avocado rating scale of skin colour (White *et al.*, 2009)

Rating scale	Skin colour
1	Emerald green: typical of freshly harvested early season fruit
2	Forest green: fruit is not shiny
3	Some black on green: fruit is mainly green but is beginning to darken in places (approximately 25% coloured)
4	Some green on black: fruit is mainly dark but with some portions still green (approximately 75% coloured)
5	Purple: skin is completely coloured
6	Black

Cox *et al.* (2004) indicated alterations in pigments throughout the ripening process of 'Hass' avocados. They found that the colour changes during ripening and late-season colour development in 'Hass' avocados were the result of a reduction in chlorophyll, followed by an increased synthesis of the anthocyanin cyanidin 3-O-glucoside. These pigment changes are closely linked to skin colour changes, which was influenced by ripening temperature and environmental factors.

2.3.3 Firmness of avocado fruits

Woolf *et al.* (2009) conducted a study examining the influence of 'Hass' avocado ripeness on quality and oil yield. Three different ripeness levels (minimally ripe, fully ripe, and overripe) of 'Hass' avocado fruits were processed, with corresponding hand firmness ratings of four, five, and six, respectively (White *et al.*, 2009). The results revealed that the cold-pressed oil yield increased from 7.0% to 8.5% to 11.04% (g oil/100 g wet flesh) as the fruit ripeness increased (Table 2.2). However, as fruit ripeness increased, the oil quality diminished, as indicated by the rise in FFA% (percentage free fatty acid) from 0.029% to 0.127% w/w (as oleic acid).

Table 2.2: The influence of 'Hass' avocado fruit ripeness on oil yield and %free fatty acids (%FFA) (Woolf *et al.*, 2009).

Ripeness	**4**	**5**	**6**
Oil yield % **(g oil/100g flesh tissue)**	7.0±0.84	8.5±0.22	11.04±0.33
% FFA (w/w)	0.029±0.017	0.093±0.021	0.127±0.037

Firmness is a very important quality parameter cold-pressed avocado oil extraction. Previous research has shown that more cell rupture occurred in softer avocados during cold-pressed extraction, which gave a higher oil yield (Yang *et al.*, 2019). White *et al.* (2009) has prescribed the hand firmness guide for avocado as it is shown in Table 2.3.

Table 2.3: Avocado hand firmness guide (White *et al.*, 2009).

0	Hard, no 'give' in the fruit
1	Rubbery, slight 'give' in the fruit
2	Sprung, can feel the flesh deform by 2-3 mm (1/10 inches) under extreme thumb force
3	Softening, can feel the flesh deform by 2-3 mm (1/10 inches) with moderate thumb pressure
4	Firm-ripe, 2-3 mm (1/10 inches) deformation achieved with slight thumb pressure. Whole fruit deforms with extreme hand pressure
5	Soft-ripe, whole fruit deforms with moderate hand pressure
6	Overripe, whole fruit deforms with slight hand pressure
7	Very overripe, flesh feels almost liquid

There is another method using a penetrometer, which was designed to penetrate the fruit after applying a certain pressure, although this is destructive in that some of the skin should be removed before testing (White *et al.*, 2009). For intact fruits, it is preferable to use a Firmometer for measurement. It is noted that the Firmometer value rises with the increase in softness (Woolf *et al.*, 2009).

2.3.4 Ethylene treatment to ripen avocados

The expression of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) genes in a plant, is typically subject to distinct and precise regulation by various hormonal, environmental and developmental signals (Kende, 1989). Small amounts of ACC and low ACS activity have been found in pre-climacteric avocado fruit, which increases significantly during the climacteric phase and reaches a maximum level shortly before the ethylene peak (Sitrit *et al.*, 1986). The activity of ACC oxidase (ACO) in avocados significantly increases during the ethylene surge associated with ripening (Owino *et al.*, 2002). Avocados are often ripened with ethylene before oil extraction to increase the oil yield (Pedreschi *et al.*, 2016; Woolf *et al.*, 2009). Several factors influence the impact of ethylene on avocado fruits, including fruit maturity, ethylene concentration, treatment duration, treatment temperature, carbon dioxide levels and storage temperature (White *et al.*, 1999). Avocados are normally treated with 100 $\mu L \cdot L^{-1}$ of ethylene at a temperature range of 20-22 °C for 24 hours, after which the fruits usually takes about four to six days to ripen when kept at the same temperature range (20-22 °C) (Hofman *et al.*, 2013).

2.4 Components of avocado fruit

The avocado fruit consists of three main parts: pulp (mesocarp), skin (exocarp) and seed (stone). Depending on different cultivars, the pulp of avocado fruit contributes to 50–80% of the fruit weight, whilst seed accounts for 10–25% (Lewis, 1978). Based on fresh weight, ripe ‘Hass’ avocado typically comprises roughly 68% flesh, 18% seed, and 14% skin on average (Wong *et al.*, 2008). There has been a significant surge in interest in utilizing avocado as a functional ingredient in food products. This heightened interest primarily stems from the presence of various bioactive compounds within avocados. These compounds encompass unsaturated fatty acids, dietary fibre, as well as essential vitamins such as vitamin C, B, and E. Additionally, avocados are a source of various pigments including carotenoids, chlorophylls, and the skin and seed contain anthocyanins and can contain a substantial amount of phenolic compounds (Kosińska *et al.*, 2012; Saavedra *et al.*, 2017; Wang *et al.*, 2010).

2.4.1 Avocado Pulp

Currently, the majority of chemical and bioactivity research has primarily concentrated on the avocado's pulp or flesh, also known as the mesocarp. There is approximately 30% (g oil/100g fresh flesh) oil in the pulp of avocados when mature, which is abundant in monounsaturated fatty acids. These fatty acids have been suggested to offer advantageous effects on risk factors associated with cardiovascular disease (CVD) (Colette *et al.*, 2003; Pieterse *et al.*, 2005). Moreover, the pulp of the avocado is a rich source of various bioactive phytochemicals, which include polyphenols (Table 2.4), carotenoids (lutein, α-carotene, cryptoxanthin and zeaxanthin) (Table 2.5), vitamins B, C, E, β-sitosterol, D-mannoheptulose and persenone A and B (Ashton *et al.*, 2006; Di Stefano *et al.*, 2017; Jimenez *et al.*, 2021; Santana *et al.*, 2019). These components have exhibited notable activities, including antitumor, antifungal, and antioxidant properties (Lu *et al.*, 2005). In laboratory studies, avocado pulp has been shown to possess inhibitory effects on cancer cell growth in vitro. The active compounds responsible for these effects are suggested to be lutein, along with other carotenoids and tocopherols (Lu *et al.*, 2009). Avocado pulp is also well-known for its advantageous effects on the human skin. Combined with the unsaponifiable fraction from soybean oil, the unsaponifiable fraction of avocado pulp can be utilized for the treatment of osteoarthritis. Plus, recent research has also reported the anti-inflammatory and anti-carcinogenic effects of this combination (Altinel *et al.*, 2007; Boileau *et al.*, 2009; Ding *et al.*, 2007; Kawcak *et al.*, 2007; Lu *et al.*, 2005).

Table 2.4: Contents of individual phenolic compounds of ripe 'Hass' avocado pulp

Phenolic compounds	Solubility	Quantity	Reference
3.4 di-OH-p-phenylacetic acid	water soluble	0.51 mg/100g DW	(Santana *et al.*, 2019)
4-hydroxybenzoic acid	water soluble	0.27 mg/kg fresh sample	(Di Stefano *et al.*, 2017)
Benzoic acid	water soluble	0.99 mg/kg fresh sample	(Di Stefano *et al.*, 2017)
Caffeic acid	water soluble	0.20 mg/kg fresh sample	(Di Stefano *et al.*, 2017)
Epicatechin	lipid soluble	6.28 mg/kg fresh sample	(Di Stefano *et al.*, 2017)
Ferulic acid	lipid soluble	0.72 mg/100g DW	(Santana *et al.*, 2019)
		2.29 mg/kg fresh sample	(Di Stefano *et al.*, 2017)
Gallic acid	lipid soluble	0.52 mg/100g DW	(Santana *et al.*, 2019)
Gentistic acid	water soluble	0.24 mg/kg fresh sample	(Di Stefano *et al.*, 2017)
p-Coumaric acid	water soluble	1.29 mg/100g DW	(Santana *et al.*, 2019)
		3.64 mg/kg fresh sample	(Di Stefano *et al.*, 2017)
Quercetin	water soluble	0.11 mg/100g DW	(Santana *et al.*, 2019)
Sinapinic acid	water soluble	0.43 mg/kg fresh sample	(Di Stefano *et al.*, 2017)

DW = dry weight

Table 2.5: Contents of individual pigments of ripe 'Hass' avocado pulp

Pigments	Solubility	Quantity	Reference
Lutein	lipid soluble	0.41 µg/g FW	(Ashton *et al.*, 2006)
β-carotene	lipid soluble	0.10 µg/g FW	(Ashton *et al.*, 2006)
Neoxanthin	lipid soluble	1.21 µg/g FW	(Ashton *et al.*, 2006)
violaxanthin	lipid soluble	0.49 µg/g FW	(Ashton *et al.*, 2006)
α-carotene	lipid soluble	0.05 µg/g FW	(Ashton *et al.*, 2006)
Antheraxanthin	lipid soluble	0.05 µg/g FW	(Ashton *et al.*, 2006)
Zeaxanthin	lipid soluble	0.02 µg/g FW	(Ashton *et al.*, 2006)
Chlorophyll a	lipid soluble	3.58 µg/g FW	(Ashton *et al.*, 2006)
Chlorophyll b	lipid soluble	0.45 µg/g FW	(Ashton *et al.*, 2006)

FW = fresh weight

2.4.2 Avocado Skin

The avocado skin (peel) is composed of carbohydrates (62-73.3%), lipids (4.4-9.1%)，proteins (4-8.3%) and an abundant amount of dietary fibre (nearly 50%), which is also rich in bioactive compounds (Colombo & Papetti, 2019). Compared to other fruit products, the avocado skin is known for its high phenolic content (Table 2.6) and antioxidant properties, which are notably more abundant in avocado skin (Adikaram *et al.*, 1992; Morais *et al.*, 2015). Phenolic phytochemicals have been associated with a range of health benefits, including anti-tumour, anti-aging, anti-inflammatory, antioxidant, and anti-diabetic effects (Ahangarpour *et al.*, 2019; Saavedra *et al.*, 2017). Because of their capacity to protect against oxidation and colour degradation, as well as their ability to inhibit the growth of bacteria and fungi, these compounds find extensive use in food industry (Kosińska *et al.*, 2012; Rodríguez-Carpena *et al.*, 2011). Consequently, extracting these bioactive substances from avocado skin holds potential to yield new products with enhanced value and better utilization of by-products from the avocado processing industry (Sadiye, 2021).

Table 2.6: Contents of individual phenolic compounds of ripe 'Hass' avocado skin

Phenolic compounds	Solubility	Quantity	Reference
5-O-caffeoylquinic acid	lipid soluble	81.8 µg/g DW	(Kosińska *et al.*, 2012)
		22.7 mg/g extract[a]	(Melgar *et al.*, 2018)
4-O-Caffeoylquinic acid	lipid soluble	20.2 mg/g extract[a]	(Melgar *et al.*, 2018)
Quercetin-3,4′-diglucoside	water soluble	46.1 µg/g DW	(Kosińska *et al.*, 2012)
Quercetin-3-O-arabinosyl-glucoside	water soluble	80.4 µg/g DW	(Kosińska *et al.*, 2012)
Quercetin 3-O-rutinoside	water soluble	23.8 µg/g DW	(Kosińska *et al.*, 2012)
Quercetin 3-O-galactoside	water soluble	31.2 µg/g DW	(Kosińska *et al.*, 2012)
Quercetin-3-O-glucoside	water soluble	1.2 mg/g extract[a]	(Melgar *et al.*, 2018)
Quercetin derivative (II)	lipid soluble	62.5 µg/g DW	(Kosińska *et al.*, 2012)
Quercetin-dihexoside	lipid soluble	1.4 mg/g extract[a]	(Melgar *et al.*, 2018)
Quercetin-glucoronide	lipid soluble	1.2 mg/g extract[a]	(Melgar *et al.*, 2018)
Quercetin-pentoside-hexoside	lipid soluble	1.5 mg/g extract[a]	(Melgar *et al.*, 2018)
Quercetin-hexoside	lipid soluble	1.1 mg/g extract[a]	(Melgar *et al.*, 2018)
Quercetin-rhamnoside-hexoside	water soluble	1.2 mg/g extract[a]	(Melgar *et al.*, 2018)
Quercetin-rhamnoside-pentoside	water soluble	1.0 mg/g extract[a]	(Melgar *et al.*, 2018)
Isorhametin-glucuronide	water soluble	1.1 mg/g extract[a]	(Melgar *et al.*, 2018)
catechin	water soluble	148.8 µg/g DW	(Kosińska *et al.*, 2012)
Epicatechin	water soluble	46.5 mg/g extract[a]	(Melgar *et al.*, 2018)
Procyanidin dimer B (I)	water soluble	135.4 µg/g DW	(Kosińska *et al.*, 2012)

Procyanidin dimer A	water soluble	26.8 µg/g DW	(Kosińska *et al.*, 2012)
Procyanidin dimer B (II)	water soluble	55.1 µg/g DW	(Kosińska *et al.*, 2012)
B-type (epi)catechin dimer	water soluble	34.1 mg/g extract[a]	(Melgar *et al.*, 2018)

[a] reported for ethanolic extracts.

DW = dry weight

Table 2.7: Contents of individual pigments of ripe 'Hass' avocado skin

Pigments	Solubility	Quantity	Reference
Lutein	lipid soluble	20.54 µg/g FW	(Ashton *et al.*, 2006)
β-carotene	lipid soluble	12.53 µg/g FW	(Ashton *et al.*, 2006)
Neoxanthin	lipid soluble	5.52 µg/g FW	(Ashton *et al.*, 2006)
violaxanthin	lipid soluble	4.49 µg/g FW	(Ashton *et al.*, 2006)
α-carotene	lipid soluble	3.87 µg/g FW	(Ashton *et al.*, 2006)
Antheraxanthin	lipid soluble	1.04 µg/g FW	(Ashton *et al.*, 2006)
Zeaxanthin	lipid soluble	0.92 µg/g FW	(Ashton *et al.*, 2006)
Chlorophyll a	lipid soluble	23.00 µg/g FW	(Ashton *et al.*, 2006)
		21.0 µg/g FW	(Wang *et al.*, 2010)
		0.36 mg/kg FW	(Cox *et al.*, 2004)
Chlorophyll b	lipid soluble	8.55 µg/g FW	(Ashton *et al.*, 2006)
		20.2 µg/g FW	(Wang *et al.*, 2010)
		0.16 mg/kg FW	(Cox *et al.*, 2004)
Pheophytin a	lipid soluble	0.10 µg/g FW	(Ashton *et al.*, 2006)
Pheophytin b	lipid soluble	0.05 µg/g FW	(Ashton *et al.*, 2006)

FW = fresh weight

2.4.3 Avocado Seed

Avocado seeds are regarded as waste by-products in avocado processing industries. The limited utilization of this by-product has resulted in significant environmental pollution (Figueroa *et al.*, 2018). The fresh seed weight of 'Hass' avocado seed contains approximately 52.7% to 54.1% moisture, 27.5% starch, 2.4 to 2.5% protein, 1.2% ash and 0.5% fat, notably lower in fat compared to the pulp (Dabas *et al.*, 2013; Pahua-Ramos *et al.*, 2012). Recent studies have indicated that avocado seeds have potential benefits in managing diabetes, inflammatory conditions, hypertension, hypercholesterolemia (Dabas *et al.*, 2013; del Refugio Ramos *et al.*, 2004). Various reports of bioactive compounds in avocado seed have been reported by Wang *et al.* (2010), Jimenez *et al.* (2021), Velderrain-Rodríguez *et al.* (2021) and Kosińska *et al.* (2012). The compounds are summarized in Tables 2.8. Compared to pulp and skin, avocado seeds are more abundant in phenolic compounds (Table 2.8), which are believed to contribute to their potential health benefits and as antioxidants in the food industry, for example, for preventing lipid oxidation (Bangar *et al.*, 2022; Moure *et al.*, 2001; Wang *et al.*, 2010).

Table 2.8: Contents of individual phenolic compounds of ripe 'Hass' avocado seed

Pigments	**Solubility**	**Quantity**	**Reference**
Caffeoylshikimic acid	water soluble	2210 µg/100g extract[a]	(Velderrain-Rodríguez *et al.*, 2021)
3-O-caffeoylquinic acid	water soluble	57.5 µg/g DM	(Kosińska *et al.*, 2012)
		176.20 µg/100g extract[a]	(Velderrain-Rodríguez *et al.*, 2021)
3-O-p-coumaroylquinic acid	water soluble	13.6 µg/g DM	(Kosińska *et al.*, 2012)
Catechin	water soluble	280.50 µg/100g extract[a]	(Velderrain-Rodríguez *et al.*, 2021)
		24.3-2,000 mg/100g	(Jimenez *et al.*, 2021)
Caffeic acid	water soluble	13.7-22.5 mg/100g	(Jimenez *et al.*, 2021)
Epicatechin	lipid soluble	360.00 µg/100g extract[a]	(Velderrain-Rodríguez *et al.*, 2021)
		1,106-2,906 mg/100g	(Jimenez *et al.*, 2021)
Catechin/epicatechin gallate	lipid soluble	152.8 µg/g DM	(Kosińska *et al.*, 2012)
Kaempferol	lipid soluble	10.74 mg/100g	(Jimenez *et al.*, 2021)
Procyanidin dimer (type B)	lipid soluble	207.70 µg/100g extract[a]	(Velderrain-Rodríguez *et al.*, 2021)
Procyanidin trimer A (I)	water soluble	81.7 µg/g DM	(Kosińska *et al.*, 2012)
		231.40 µg/100g extract[a]	(Velderrain-Rodríguez *et al.*, 2021)
Procyanidin trimer A (II)	water soluble	89.3 µg/g DM	(Kosińska *et al.*, 2012)

Salidroside	water soluble	148.40 µg/100g extract[a]	(Velderrain-Rodríguez *et al.*, 2021)
Trans-5-O-caffeoyl-D-quinic acid	water soluble	163-574 mg/100g	(Jimenez *et al.*, 2021)
Vanillic acid	lipid soluble	286 mg/100g	(Jimenez *et al.*, 2021)

[a] reported for ethanolic extracts.

2.5 Avocado oil

Extra-virgin avocado oil is a desirable viscous edible oil with an attractive dark green hue, attributed to its carotenoid and chlorophyll content. It possesses a unique mild flavour and is extracted from avocado fruits primarily through cold pressing methods (Cervantes-Paz & Yahia, 2021). Importantly, it maintains its natural composition without any chemical additives or additional processing steps that would modify its original qualities (Permal *et al.*, 2020; Wang *et al.*, 2020). Avocado oil stands out as a premium oil known for its high quality, and notable colour, texture and flavour (Wong *et al.*, 2010). Unlike other edible oils derived from oleaginous fruits, avocado oil is extracted from the mesocarp tissue that surrounds its seed, the seed is not generally crushed during extraction like olive oil extraction.

Crude avocado oil is of lower quality compared to extra-virgin cold-pressed avocado oil but it shares similar fatty acid composition and viscosity characteristics with extra-virgin avocado oil (Woolf *et al.*, 2009). However, its visual appearance is less appealing due to its extraction from low-quality avocado fruits, which include the pulp and sometimes together with the seed and skin. Processing poor-quality fruits results in oils that are yellowish-green colour in the crude avocado oil (Flores *et al.*, 2019). Compared to crude avocado oil, refined avocado oil is odourless, light in colour, has no waxes and has low levels of free fatty acids (Cervantes-Paz & Yahia, 2021). Crude avocado oil is refined before it is used as a food grade oil. The refining process includes several steps including degumming, neutralization, bleaching, deodorizing and winterizing. These processes help to improve the oil quality, remove impurities, and enhance its overall stability and sensory properties (Flores *et al.*, 2019; Woolf *et al.*, 2009).

There is no current globally standardized criteria for avocado oil so far. The proposed new Codex standard for refined avocado oil are nearing finalization (Codex Alimentarius Commission, 2023) although separate criteria for virgin/extra virgin avocado oil still require further development (Green & Wang, 2023).

2.5.1 History and development of avocado oil

Wong *et al.* (2014) reported in 2014 that the avocado production had increased by 52% since 1999. Based on data from FAOSTAT (2024), the estimated global production of avocados in 2022 reached 8,978,275 metric tonnes, representing a 4.8% increase from the 8,570,284 tonnes produced in 2021. This rise in production has led to an increased supply of reject-grade fruits for which growers are hoping to find a market for. With the development of high pressure processing technologies, the competition between culinary oil and guacamole production to use these reject fruits has increased (Requejo-Tapia, 1999). Depending on the time of harvest, the oil content of 'Hass' avocado flesh can be up to 32% based on its fresh weight at late season, although the average oil content during mid-season is typically around 18% (Wong *et al.*, 2014).

The major manufacturers of avocado oil around the world are Chile, South Africa, the United States, Mexico and New Zealand. In 2016, the American continent was the largest market region for avocado oil, accounting for 600,000 tons (68.8%) of the global market. Asia and Europe followed with 432,000 tons (22.6%) and 193,000 tons (8.6%), respectively (Cervantes-Paz & Yahia, 2021).

Regarding the total world production of avocado oil, accurate statistics have not been well documented. However, according to the report from Varsha (2024), the avocado oil market achieved a valuation of $USD 0.49 billion in 2021. With the increase of plant-based products in demand, the avocado oil market is considered to keep growing.

As for New Zealand, about 3% of total avocados have been utilized for producing oil between 2008 and 2009 (Wong *et al.*, 2010). Due to international demand 217.3 tons of the avocado oil was exported and 150,000 litres of the oil exported was extra-virgin grade (Wong *et al.*, 2014).

In the year 2000, New Zealand companies initiated the commercial manufacturing of cold-pressed avocado oil for the culinary market. They utilized the pulp of ''Hass'' avocados as the raw material and employed an aqueous extraction process to produce avocado oil (Woolf *et al.*, 2009).

2.6 Extraction of avocado oil

The mesocarp of the avocado flesh consists of idioblast and parenchyma cells (Tan, 2019). As it is shown in Figure 2.2, some small oil bodies sit inside the parenchyma cells within the mesocarp tissue surrounded by a thin cellulose wall (Yang, 2019), while large oil sacs are stored in idioblasts covered by a robust cellular wall comprised of a cellulosic wall, suberin layer and tertiary wall (Platt & Thomson, 1992). The main source of oil is obtained from ripe avocado fruit parenchyma cells, primarily due to the increased activity of cellulase enzymes and polygalacturonase, leading to breaking down the parenchyma cell walls and releasing the oil bodies (Platt-Aloia *et al.*, 1980).

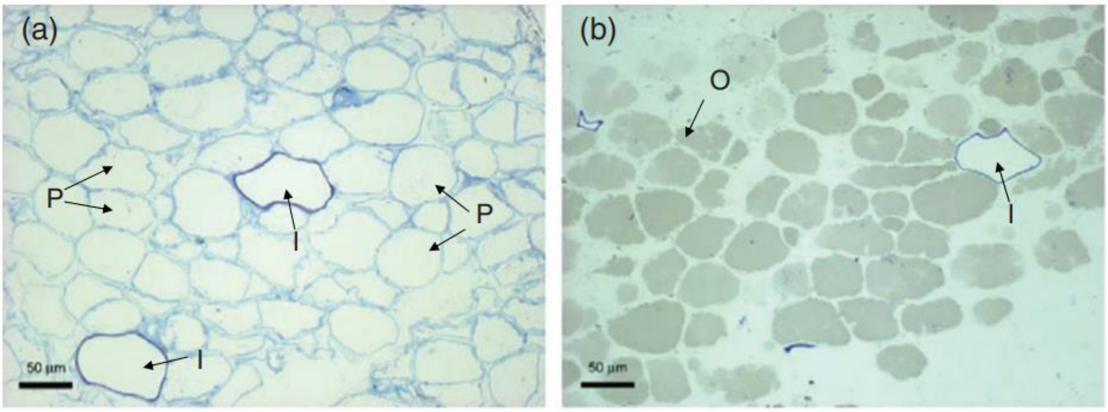

Figure 2.2. Microstructure of intact 'Hass' avocado flesh (a, b). P, parenchyma cells; I, idioblast cells; O, oil droplet(s) from parenchyma cells (Yang *et al.*, 2018)

To facilitate the release of oil bodies and achieve higher yields, it is generally achieved by thermal, mechanical and enzymatic treatments, as well as the utilization of solvents (Cervantes-Paz & Yahia, 2021). Yang *et al.* (2021) reported that after grinding most of the parenchyma cell walls are broken and higher yields were obtained with longer malaxing and higher temperatures. Yang *et al.* (2018) reported that the idioblast cells are not disrupted during grinding or malaxing and stay intact to be removed in the waste stream.

Earlier published information reported that avocado oil was obtained from dried fruit tissue using organic solvents (Human, 1987). However, mechanical techniques are also applied when solvents and fruit drying technology are not readily available (Costagli & Betti, 2015). Two approaches are used for oil extraction, solvent-based extraction or mechanical cold-pressed aqueous extraction with centrifugation (Permal *et al.*, 2020; Qin & Zhong, 2016; Wong *et al.*, 2014).

Extraction of extra virgin avocado oil to be used as a culinary oil is recovered from the flesh of the ripe avocado using a mechanical aqueous extraction process (Yang *et al.*, 2019). This process is well established in the avocado oil industry. To obtain the highest oil yield from the mesocarp flesh, the skin and stones of the avocado are first removed before grinding of the flesh (Yang, 2019). As the cellular structure (parenchyma cells) of higher maturity fruit is more easily disrupted and grinding can further improve the cell disruption, this results in higher oil yield with more mature fruit (Yang *et al.*, 2020). During this process normally only 5 - 10% of the skin is included with the pulp during malaxing (Ashton, 2005).

2.6.1 Commercial aqueous extraction systems

The commercial manufacturing of avocado oil is based on the continuous modern extraction process used for extra virgin olive oil. Only mechanical processes are used; involving rinsing, grinding, malaxing (mixing slowly) and centrifugation. The malaxing temperature of avocado flesh is maintained between 45 to 50°C, meanwhile the time of malaxing can range from 60 to 120 minutes depending on the time of the season (Wong *et al.*, 2014). Yang *et al.* (2021) reported the effect of malaxing temperature on oil yield and found 50°C gave higher oil yields than at 30°C.

Aqueous extraction methods have demonstrated their effectiveness and commercial viability in the production of avocado oil (Cervantes-Paz & Yahia, 2021). This process involves enzymatic or mechanical breakdown of cell walls in the plant tissues to release lipids, followed by oil-water separation through centrifugation or gravity setting. The combination of aqueous oil extraction and centrifugation can yield high-quality oils with distinctive and appealing organoleptic qualities, all achieved without the use of solvents. There are three primary categories of aqueous separation methods, which are defined by the method of cell wall destruction and the driving force for separating the oil layer (Qin & Zhong, 2016).

Enzymatically assisted centrifugation separation relies on the use of exogenous enzymes together with endogenous cellulolytic enzymes, which hydrolyse and degrade cell walls, facilitating oil release from the cells (Sharma *et al.*, 2015). Various enzymes such as pectinases, α-amylase, proteases, cellulases, and pectolytic enzymes can be added. Factors like enzyme type and concentration, reaction temperature, reaction time, and paste-to-water ratio significantly influence oil extraction yield (Qin & Zhong, 2016). Enzyme-assisted centrifugation has been shown to enhance avocado oil yield compared to non-enzyme-assisted methods (Roda *et al.*, 2019).

Mechanically assisted centrifugation is another approach, wherein mechanical force (e.g., grinding) ruptures cells containing oil droplets. Centrifugation then separates the released oil (Qin & Zhong, 2016). To prevent emulsification of oil with water and other cellular substrates, some methods are normally applied before centrifugation including adding demulsifying agents, adjusting pH values, and heating. The addition of inorganic salts, such as $CaSO_4$, $CaCO_3$ and NaCl, has also been found to promote oil-water separation (Bizimana *et al.*, 1993; Werman & Neeman, 1987). However, lipoxygenase activity, which can degrade unsaturated fatty acids, may still be present in crude avocado oil extracted using this method. To address this issue, moderate heating and electric field treatments have been explored to minimize the oxidation of unsaturated fatty acids and preserve oil quality (Ariza-Ortega *et al.*, 2014). Plus, there is a mechanically assisted hot water separation method, where the destruction of cell walls is achieved through mechanical force and hot water used as a separating agent (Qin & Zhong, 2016).

Not like solvent-based and pressing extraction methods, aqueous separation techniques have the minimal need to remove excess water from the fresh pulp (Yang *et al.*, 2021; Yang *et al.*, 2018). The malaxing step and the use of centrifugal force not only aids in oil-water separation but also results in a higher-quality product, simplifying subsequent refining processes (Pérez-Saucedo *et al.*, 2021). The combination of aqueous oil extraction and centrifugation can yield high quality oils featuring distinctive and appealing sensory attributes, all achieved without the reliance on solvents. Through effective processing oversight, it is also possible to minimize the generation of waste streams (Wong *et al.*, 2014).

2.6.2 Pressing extraction

Oil extraction from avocado pulp can be achieved through pressing, which involves squeezing the oily material using a screw press or hydraulic press. This method is commonly used for oilseed materials like sesame that have high oil content. Avocado pulp, on the other hand, contains more moisture (about 77%), necessitating different pretreatment methods before pressing, which can include slicing and drying, microwave-oven drying, or the addition of solid additives (Qin & Zhong, 2016).

Traditional drying methods, such as sun-drying and oven-drying, are time-consuming and may result in poor oil quality (Qin & Zhong, 2016). In contrast, microwave-oven drying not only reduces drying time but also disrupts cell structures, which can be beneficial for oil extraction. Factors like the quantity of samples, microwave energy intensity, and exposure time influence oil extraction yield. High microwave energy, often accompanied by temperatures exceeding 100°C, can negatively impact oil cell structures and reduce yield. Optimal results are typically achieved with an energy input of around 1.89 kJ/g, which disrupts cell integrity (Moreno *et al.*, 2003).

Another approach to increase oil extraction yield is by adding solid additives to reduce avocado pulp's viscosity and moisture. These additives should have specific granularity and hardness, be non-toxic, and insoluble in water and oil. For instance, rice, sorghum rice, and grains of sand are more effective additives than rice husk, sugar, and salt (Satriana *et al.*, 2019). After mixing the additives with avocado pulp, heating is required to facilitate cell wall disruption during extrusion, reducing cellular oil viscosity and increasing oil yield. Moderate heating also helps inactivate lipases, preventing the hydrolysis of avocado oil during storage and squeezing (Qin & Zhong, 2016).

2.6.3 Solvent extraction

Organic solvent extraction is a common method for oil separation from various sources, including avocados. It involves slicing, drying, and grinding the avocado fruit, followed by the extraction of oil using solvents such as hexane and acetone (Moreno *et al.*, 2003). The choice of solvent significantly impacts the extraction yield, with hexane resulting in rough cell structure modifications and acetone causing significant deformation and oil retention within the idioblast cells (Ortiz *et al.*, 2004). However, despite higher yields, organic solvent extraction methods have environmental concerns and can leave solvent residues in the final products, limiting their use in the food and pharmaceutical industries (Qin & Zhong, 2016).

Oven-drying and freeze-drying are two common moisture reduction methods, with freeze-drying yielding more brittle and powdery material (Moreno *et al.*, 2003). However, the choice of drying method affects oil extraction yield, with freeze-drying proving more effective. Nevertheless, freeze-dried material may still contain lipase enzymes, impacting oil quality. Factors like drying costs, efficiency, and the concentration of minor compounds must be considered when evaluating oil quality and oxidative stability in the industry (Mostert, 2008).

2.7 Laboratory extraction systems

2.7.1 Soxhlet extraction

Soxhlet extraction is one of the most widely used methods for extracting lipids from solid samples. The traditional Soxhlet technique has found regular use in nearly every analytical laboratory since its discovery in 1879 (Zygler *et al.*, 2012). Nowadays, the Soxhlet extraction method continues to serve as the benchmark against which the efficiency of contemporary extraction techniques is assessed (Manirakiza *et al.*, 2001). In this method, as shown in Figure 2.3, a porous thimble containing the solid sample is positioned within the primary chamber of the Soxhlet extractor. By employing a condenser and a siphon side arm to enable solvent reflux through the thimble, the extraction cycle is typically repeated multiple times. Soxhlet extraction is a robust and well-established procedure that allows for extraction without constant supervision. Nonetheless, it necessitates an extended extraction duration and the consumption of a substantial amount of solvent (Kim *et al.*, 2012).

Ramluckan *et al.* (2014) has demonstrated that when it comes to single solvent extractions, chloroform, ethanol, and hexane yield the highest lipid quantities. It was determined that an extraction duration of three hours provides the best lipid yields. Therefore, employing longer extraction periods would result in unnecessary rises in extraction expenses. It is important to note that the presence of moisture in food significantly impacts the lipid extraction yield (Lin *et al.*, 2004). High levels of water content in the food hinder the interaction between lipids and nonpolar solvents (e.g., n-hexane), leading to lower lipid yields (de Jesus *et al.*, 2019). Consequently, to achieve a more precise total oil content, it is advisable to minimize the moisture in the sample when using non-polar solvents (Saini *et al.*, 2021).

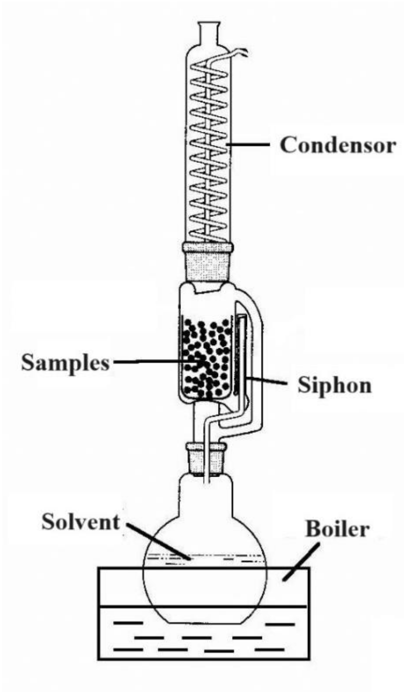

Figure 2.3: Soxhlet extraction apparatus modified drawing based on Dean (2010)

2.7.2 Accelerated Solvent Extraction (ASE)

Accelerated solvent extraction (ASE), also known as pressurized liquid extraction (PLE) and pressurized fluid extraction (PFE), is a method of liquid solvent extraction that employs elevated temperatures and pressures with both aqueous and organic extraction solvents, thereby preserving the solvent in its liquid form above its normal atmospheric boiling point (Giergielewicz-Możajska *et al.*, 2001; Richter *et al.*, 1996). While the initial emphasis of this method was in environmental applications, its adaptability and ease of use have demonstrated its value in laboratories for performing extractions within the food industry (Luthria *et al.*, 2019).

Since its inception in 1995, ASE has experienced significant growth in its adoption as a viable alternative to conventional extraction techniques. It utilizes the same solvents as traditional methods but at higher temperatures than what conventional techniques allow. This temperature elevation enhances the speed and efficiency of the extraction process, leading to more efficient extractions with reduced solvent usage compared to traditional methods (Dean *et al.*, 1997; Sporring *et al.*, 2005). The use of pressurized conditions ensures that the solvents remains in their liquid state even when subjected to high temperatures (Mottaleb & Sarker, 2012). Raising the solvent's temperature leads to reduced viscosity, facilitating deeper penetration into the sample matrix. Moreover, it enhances analyte diffusion from the sample matrix into the solvent and increases the overall solvent capacity(Luthria *et al.*, 2019).

During the ASE process (Figure 2.4), a solid sample is enclosed within a sample cartridge, which is filled with extraction fluid. The sample is then statically extracted at elevated temperatures (50-200 °C) and under high pressure conditions (3500-20000 kPa) for brief durations (5-10 minutes). Compressed gas is utilized to transfer the sample extract from the cell into a collection vessel. ASE is currently suitable for solid or semi-solid samples that can be retained within the cell during the extraction process (Richter *et al.*, 1996).

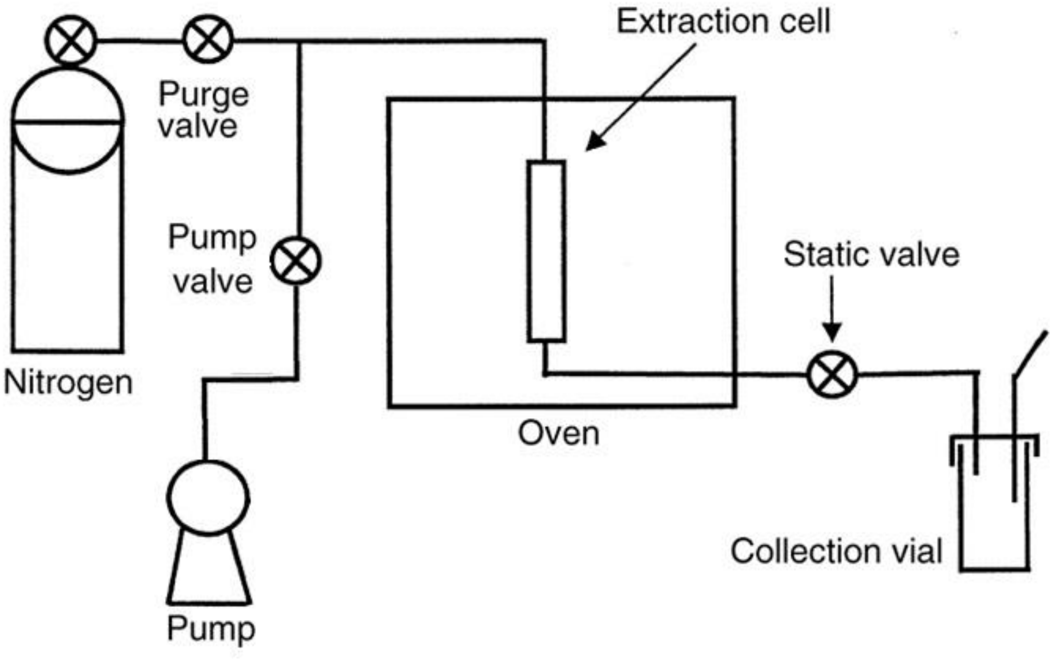

Figure 2.4: Diagram of the accelerated solvent extraction (ASE) system modified drawing based on Luthria *et al.* (2019).

2.7.3 Microwave-assisted extraction

Microwave-assisted extraction (MAE) is an innovative technique for extracting valuable components from diverse materials by utilizing microwave energy, which provides a swift and uniform delivery of energy to both solid plant matrix and solvent. This results in efficient and homogeneous heating of solid matrix and solvent, enabling the extraction of target compounds from the sample into the solvent. MAE is grounded in the principle of the microwave heating system's selectivity. It directly impacts polar solvents and/or materials, reducing the moisture content in sample cells (Satriana *et al.*, 2019).

MAE is recognized as one of the environmentally friendly technologies due to its ability to reduce the usage of organic solvents (Alupului *et al.*, 2012). It is worth noting that microwave energy can potentially induce quantitative modifications and lipid oxidation of fatty acids. Nevertheless, these alterations seem to be negligible under standard conditions. In MAE, irradiation leads to selective heating of the sample-solvent mixture, potentially subjecting lipids to more challenging conditions compared to certain solvent extraction methods that involve conventional heating (Sahena *et al.*, 2010).

MAE is gaining popularity as a method for laboratory oil extraction, although there is limited research on avocado oil extraction using this technique. Most studies involve using microwaves for pretreating avocado pulp before solvent extraction. Moreno *et al.* (2003) conducted a study comparing the performance of MAE in avocado oil extraction. Their results showed that a higher avocado oil yield was obtained using a combination of solvent extraction and microwave drying when compared to the traditional Soxhlet method. Additionally, it was observed that solvent extraction led to greater oil deterioration compared to microwave-assisted methods.

In a similar study by Reddy *et al.* (2012), oils produced through this technique had a high ratio of monounsaturated fatty acids to saturated fatty acids, and the quality of the oil was superior to that obtained via Soxhlet extraction. Ortiz *et al.* (2004) has demonstrated that MAE, which combines microwave treatment, squeezing, and pressing, induced minimal changes in the cell structure, resulting in improved quality and quantity of the extracted avocado oil. This method also yielded oil with high oxidative stability and low acidity, making it suitable for consumption as an edible oil (Santana *et al.*, 2015).

2.7.4 Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) has found application in the accelerated extraction of compounds from plant materials. This method is notably effective when compared to traditional extraction techniques because it enables cell disruption and molecular interactions with solvents at relatively low temperatures (Rupasinghe *et al.*, 2011). This enhanced extraction process likely occurs by intensifying mass transfer and accelerating the penetration of solvents into the cellular structures of plant materials in the presence of ultrasound. It's important to exercise precise control over the extraction temperature, given that ultrasound generates heat during the process (Wang & Weller, 2006).

Compared to traditional extraction methods, ultrasound creates disrupts cell walls and micro-fractures, facilitating the release of cell contents (Chemat *et al.*, 2004). Variations in extraction yield among different plant varieties can be attributed to disparities in material structure, rheology, hardness, and composition, resulting in varying susceptibilities to ultrasound shock waves and the likelihood of cavitation bubbles contacting the plant surface, causing micro-jetting (Li *et al.*, 2004). Utilizing ultrasound energy in the extraction process presents several advantages, including reduced thermal gradients, smaller equipment requirements, more effective mixing, lower extraction temperatures, selective extraction, quick process control response, rapid energy transfer, fast startup and increased production (Chemat *et al.*, 2008).

Ultrasound is frequently combined with traditional techniques to enhance their effectiveness and efficiency. Implementing ultrasound pretreatment on the material before extraction increases yield and reduces the required time for oil extraction (Shah *et al.*, 2005). Reddy *et al.* (2012) extracted avocado oil from dried samples using hexane as the solvent and sonicated the samples in a water bath at 60°C for 1 hour. They reported that UAE resulted in the lowest yield (54.63%) and the least reproducible results compared to the Soxhlet method, supercritical fluid extraction and MAE. Plus, Tan *et al.* (2018a) combined ultrasonic treatment and mechanical pressing when extracting avocado oil. Avocado oil extracted by this method exhibited higher iodine values but lower free fatty acid (FFA), contents slip melting points and saponification values than oils obtained via hexane solvent extraction. In another research of Tan *et al.* (2018b), found the ideal process parameters for achieving the maximum recovery of low free fatty acid (FFA) virgin avocado oil: sonication temperature of 35°C, water to powder ratio of 6 mL/g and sonication time of 30 minutes. Under these optimized conditions, the avocado oil exhibited an oil recovery rate of 72.79% and a low FFA level of 0.297%. Yang (2019) reported that ultrasound treatment at 20-25 kHz could help with the oil aggregation during avocado oil extraction, thereby improving the cold-pressed oil yield.

Ultrasound has a positive effect on the extractability of avocado oil from both malaxed and non-malaxed tissues. In a study by Martínez-Padilla *et al.* (2018), low-frequency intervention impacted oil quality, but high-frequency intervention maintained oil quality at nearly the same specific energy levels. High-frequency treatment of the tissue post-malaxation has led to extractability improvements.

2.7.6 Supercritical CO_2 Method

In recent times, supercritical fluid extraction, using supercritical carbon dioxide (SC-

CO_2) as a green solvent, has gained attention for separating compounds from solid matrices. This extraction method relies on the use of supercritical fluids, which, under specific conditions, exhibit properties between liquids and gases. SC-CO_2 is both environmentally safe and leaves no solvent residues in the final product, making it an attractive alternative to traditional organic solvents (Qin & Zhong, 2016). Furthermore, the solubility of desired compounds and other lipid-soluble bioactive components can be controlled through adjustments in pressure and temperature during SC-CO_2 extraction. This method can minimize the co-extraction of chlorophyll and reduce unsaponifiable matter in avocado oil, leading to enhanced oil quality (Botha & McCrindle, 2002). Additionally, the separated unsaponifiable matter finds value in cosmetic and pharmaceutical industries. SC-CO_2 extraction serves a dual role of extraction and purification, making it advantageous for industrial avocado oil extraction (Mostert *et al.*, 2007).

Supercritical CO_2 is a completely benign gas that transforms into a potent solvent when subjected to elevated temperature and pressure above its critical point (Valdés *et al.*, 2019). Extraction with SC-CO_2 is most effective at a pressure of 400 bar. The addition of ethanol as a co-solvent enhances the extraction of residual oil, particularly benefiting the extraction of a tocopherol-enriched fraction (Corzzini *et al.*, 2017).

Flores *et al.* (2019) has reported the quality assessment of avocado oil extracted through cold pressing, Soxhlet, and SC-CO_2 methods, evaluating the oil's quality based on parameters including peroxide index, free fatty acid titration, iodine index, specific gravity and saponification, following the American Oil Chemists' Society (AOCS) standards. Supercritical fluid extraction yielded the highest yields and superior quality. Oils extracted using SC-CO_2 exhibited lower acidity levels (0.48%), reduced oxidation of unsaturated fatty acids (16.87 meqO_2/kg), and higher iodine index (80.18 cgI_2/g) when compared to other methods. Though cold-pressed extraction yielded better results in terms of vitamin E content compared to SC-CO_2.

In the context of pressurized fluid extraction, the use of liquefied gas of compressed oil (LPG), composed of n-butane, propane, ethane, isobutane, and other hydrocarbons, displayed superior oil extraction performance in less time and with reduced solvent consumption compared to the SC-CO_2 method. However, avocado oil obtained using compressed LPG contained higher concentrations of stigmasterol, lycopersene, linoleic acid, oleic acid, and palmitic acid. On the other hand, SC-CO_2 extraction resulted in a higher yield of antioxidant activity, determined using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical assay (Abaide *et al.*, 2017).

2.8 Important quality parameters of avocado oil

Quality parameters are essential not only for avocado oil but also for all vegetable-fruit oils, serving both legislative and consumer safety purposes. Traditional oil extraction methods typically involve the application of heat. While heat application offers several desirable technological advantages, it can also lead to undesired reactions. Oils are sensitive to heat, which can result in quality degradation due to chemical instability (Tan & Man, 1999; Van Boekel, 1996). A higher percentage of free fatty acids indicates hydrolysis reactions have occurred during processing and high peroxide values increase if the oil is exposed to oxygen and begins to oxidise (Miyashita & Takagi, 1986).

2.8.1 Free fatty acid (FFA)

The suitability and quality of plant oils are directly impacted by the level of free fatty acids (FFA). A relatively high amount of FFA may occur in avocado oil due to inappropriate extraction techniques (Santana *et al.*, 2015). Free fatty acids are produced when the fatty acid molecules are hydrolysed and released from the glycerol backbone of triglycerides. Any free fatty acids present can function as pro-oxidants within plant oils, which lead to oxidation reactions and undesirable oxidized oils, resulting in undesirable oil flavour and odour (Chew *et al.*, 2016). Certain traditional extraction methods, for example Soxhlet extraction, can exacerbate this issue (Frega *et al.*, 1999).

The use of high temperatures during the oil extraction process may lead to the degradation of unsaturated fatty acids, consequently compromising the overall quality of the oil (Latif & Anwar, 2009). Lipid alteration often involves lipid hydrolysis, leading to the generation of FFAs through chemical or enzymatic processes, this occurs when water is present in the oil. This phenomenon is particularly note-worthy in lipid-containing matrices with water, such as virgin olive oils and avocado oil after extraction if water is not reduced to less than 0.2% (Codex Alimentarius Commission, 2023). Some studies have explored the pro-oxidant effects of FFAs, which are thought to be linked to their carboxylic molecular groups, accelerating the decomposition of hydroperoxides (Miyashita & Takagi, 1986; Velasco & Dobarganes, 2002). Despite the distinct origins and outcomes of oxidative and hydrolytic degradation processes, they appear to interact and collectively contribute to the reduced shelf life of the oil (Frega *et al.*, 1999). Although FFA itself is non-toxic, its presence can impact food quality. Therefore, measuring FFA is essential for controlling food quality and safety (Gotoh & Wada, 2006). It is note-worthy that FFA is different with the acid value (AV), which is the quantity of potassium hydroxide needed to neutralize the free fatty acids in one gram of fat. The acid value is often transformed into free fatty acid (FFA) content by multiplying it by a factor derived from the molecular weights of the relevant fatty acid (usually oleic acid, MW = 282.4) and potassium hydroxide (56.1) (Dijkstra, 2016). The standard method to determine the free fatty acid percentage in an oil is AOCS Ca 5a-40 or ISO 660: 1996, amended 2003 (AOCS, 2017; Codex Alimentarius Commission, 2023).

2.8.2 Peroxide value (PV)

Edible oils are susceptible to oxidation during storage and processing, especially when exposed to elevated temperatures, oxygen, light, and heavy metal ions (Karami *et al.*, 2020). This oxidative process, known as autoxidation, is a key contributor to the deterioration of oils and involves a chain reaction of free radicals, encompassing initiation, propagation, and termination steps (Gordon, 2004). Hydroperoxides are the primary oxidation products and are relatively stable at room temperature, providing a valuable means to assess the primary lipid oxidation status of oil samples that haven't undergone any prior oxidation. However, hydroperoxides are susceptible to decomposition, leading to the formation of alcohols, aldehydes, ketones, and other secondary oxidation products, which impart undesirable flavours to edible oils and render them less acceptable to consumers (Gotoh & Wada, 2006; Zhang *et al.*, 2021). These compounds are formed after secondary oxidation which occurs after primary oxidation (Gordon, 2004).

Hence, measuring lipid hydroperoxides during the initial stages of oxidation is crucial for evaluating the quality and degree of oxidation of oils. The peroxide value (PV) serves as an indicator for these early oxidation stages and is widely employed to gauge the total hydroperoxide content and monitor lipid oxidation throughout the storage and processing of oils (Hori *et al.*, 2019). The PV is expressed in milliequivalents (meq) of active oxygen per kilogram of oil and plays a significant role in assessing food quality and ensuring consumer safety. When the PV is greater than a critical threshold, edible oils can develop a rancid taste and, in severe cases, may pose a risk of food poisoning (Gotoh & Wada, 2006). Consequently, determining the PV of edible oils is imperative from both food quality and human health perspectives (Bustani & Soni, 2023). The standard method for analysing PV is by AOCS Cd 8b-90 or ISO 3960: 2001(AOCS, 2017; Codex Alimentarius Commission, 2023), but other methods are also reported by Zhang *et al.* (2021) using chromatographic, spectroscopic and electrochemical approaches.

2.8.3 Specific extinction coefficient

Ultraviolet spectrophotometric analysis at wavelengths of 232 and 270 nm provides simple and valuable parameters for evaluating the oxidative status of oil, with their values expressed as specific extinction coefficients (International Olive Council, 2019a). K_{232} determined at 232nm represents the measure of primary oxidation products, specifically conjugated dienes, which form due to a shift in one of the double bonds. K_{270}, measured at 270 nm, provides an indication of the amount of conjugated trienes, encompassing primary oxidation products and secondary oxidation products like aldehydes and ketones (Angerosa *et al.*, 2006; Kiritsakis *et al.*, 2002).

2.8.4 Oxidative Stability (Rancimat)

Lipid oxidation in edible oils is a critical reaction that can lead to quality deterioration. Numerous accelerated methods have been developed to assess the resistance of edible fats and oils to oxidation. These accelerated methods involve elevated temperatures, as it is well-known that the reaction rate is exponentially related to temperature (Rustad, 2009). Among these methods, the Rancimat method has gained popularity due to its ease of use and reproducibility. As a dynamic technique, it is utilized to assess the oxidation induction time, offering more reliability, reproducibility, but also less time-consuming (Aktar & Adal, 2019). This test enables the determination of the induction time, representing the time when secondary oxidation products are produced at an accelerated rate (Farhoosh *et al.*, 2008). The Rancimat method monitors the production of volatile compounds formed during secondary oxidation. When these are formed they are condensed in water and their presence indicated by an increase in conductivity (Metrohm, 2019). The Rancimat method is usually used for assessing the oxidative stability, revealing the product's resistance to the initiation of the oxidation process characterized by free radical reactions (Aparicio *et al.*, 1999).

2.8.5 Bitterness

Research indicates that the bitterness, astringency, and pungency in olive oil are associated with its high levels of phenolic compounds (Andrewes *et al.*, 2003; Mateos *et al.*, 2004; Siliani *et al.*, 2006). Some consumers in the United States, Canada, Japan, and the European Community are not accustomed to sensory characteristics associated this phenolic compounds (Mateos *et al.*, 2004). Phenolic compounds are present in avocado skin and seed (Dabas *et al.*, 2013; Sadiye, 2021), however it has not been reported if these compounds affect the bitterness of avocado oil.

Normally avocado oil is considered to be neutral taste, but improper processing or storage could cause rancid or bitter taste (Hunter&Gather, 2021; Woolf *et al.*, 2007). However, there is no published article on bitterness in avocado oil , hence it is common to use methods designed for measuring the bitterness of olive oil. Beltrán *et al.* (2007) have suggested an easy technique for assessing the intensity of oil bitterness without the need for sensory evaluation, achieved by measuring the total phenol content. Gutiérrez Rosales *et al.* (1992) introduced a straightforward analytical approach that involves extracting the bitter compounds and measuring them through spectrophotometric determination at a wavelength of 225 nm. They reported a strong correlation with the bitter taste assessed through sensory analysis. The "bitter index" (K_{225} value) has been widely employed by numerous researchers in many publications and has been subject to critical examination in some studies. The suitability and reliability of absorbance at 225 nm have been confirmed (Beltrán *et al.*, 2007; Favati *et al.*, 2013; Inarejos-Garcia *et al.*, 2009).

2.9 Major chemical components in avocado oil

The oil extracted from 'Hass' variety avocados grown in Mexico, the United States, New Zealand and Australia has shown a notable lipid content of 62% (g/ g flesh). 'Hass' avocado oil from New Zealand displayed elevated levels of unsaturated compounds and natural pigments in comparison to oils originating from other countries (Tan *et al.*, 2017).

2.9.1 Fatty acid profile

The fatty acid profile of avocado oil (Table 2.9) is similar to that of olive oil, characterised by a high proportion of oleic acid (C18:1). It is important to note that the growing environment can have a substantial impact on the fatty acid composition (Woolf *et al.*, 2009).

Table 2.9: 'Hass' avocado oil fatty acid profile from five different countries (Woolf *et al.*, 2009)

Fatty acids (% of total)	New Zealand		Australia	Chile	Mexico	California
	Range	Mean	Mean	Mean	Mean	Mean
Palmitic acid C16:0	9.7-15.2	12.3	21.7	13.1	14.8	14.5
Palmitoleic acid C16:1	1.7-8.2	4.1	9.3	3.6	7.9	4.1
Stearic acid C18:0	0.1-0.4	0.3	0.4	0.4	0.4	0.3
Oleic acid C18:1	61.7-77.8	71.5	51.8	68.2	66.8	65.3
Linoleic acid C18:2	7.7-18.9	11.6	16.0	13.2	9.5	15.0
Linolenic acid C18:3	0.2-0.9	0.5	0.8	0.8	0.6	0.8

A typical composition of fatty acids in avocado oil comprises saturated fatty acids such as palmitic acid (C16:0) and stearic acid (C18:0), monounsaturated fatty acids like palmitoleic acid (C16:1) and oleic acid (C18:1), as well as polyunsaturated fatty acids, which encompass linoleic acid (C18:2) and linolenic acid (C18:3) (Arpaia & Eaks, 1990; Inoue & Tateishi, 1995; Ratovohery *et al.*, 1988).

2.9.2 Pigments

In the process of cold-pressed extraction, significant quantities of lipid soluble plant pigments, including chlorophylls and carotenoids, are transferred into oil. Among the carotenoids, lutein is the most prominent with concentrations of 0.5 - 3.3 µg/g oil. Lutein is well-known for its role in reducing the risk of age-related macular degeneration, thereby promoting eye health (Lu *et al.*, 2005). Avocado oil has nearly double content of lutein compared to olive oil (Criado *et al.*, 2007). Additionally, cold-pressed avocado oil contains chlorophyll (11.1–18.5 µg /gram oil), which also has potential health benefits (Ashton *et al.*, 2006; Martins *et al.*, 2023; Woolf *et al.*, 2009). Chlorophyll acts as a potent photosensitizer, having no positive impact on the stability of the oil but actively participating in any photo-oxidation that may occur. Therefore, it becomes crucial to safeguard the oil during processing and storage by minimizing its exposure to both light and oxygen (Minguez-Mosquera *et al.*, 2008).

2.9.3 Phenolics compounds

Similar to olive oil, avocado oil also contains beneficial antioxidants in the form of naturally occurring plant phenols (Chaiyasut *et al.*, 2019; Santana *et al.*, 2019; Woolf *et al.*, 2009). Compared to the pulp, the antioxidant capacity and phenolic compound content are significantly greater in avocado peel and seed (Kosińska *et al.*, 2012; Rodríguez-Carpena *et al.*, 2011; Wang *et al.*, 2010). Therefore despite having lower lipid quantities, adding the peel and seed during extraction offers the potential to reduce additional waste generation while simultaneously enhancing the antioxidant content in the resulting oil (Santana *et al.*, 2019). Santana *et al.* (2019) have also researched the effects of peeling and not peeling on the phenolic compounds and content of avocado oil (Table 2.10), which found unpeeled fruits can lead to an increase in the total phenolic content and an increase of the variety of phenolic compounds of avocado oil.

Table 2.10: Phenolic compounds in peeled and unpeeled avocado oil (Santana *et al.*, 2019)

Phenolic compounds	**Phenolic contents (mg/100g oil)**	
	Peeled	Unpeeled
Gallic acid	0.52	0.94
3,4 di-OH-p-phenylacetic acid	0.51	1.39
p-OH-benzoic acid	ND	0.17
Vanillic acid	ND	0.19
p-Coumaric acid	1.29	0.93
Ferulic acid	0.72	0.64
Quercetin	0.11	0.13
Total phenolics content	3.15	4.39

2.9.4 Sterols

Similar to cholesterol, phytosterols are naturally occurring compounds in plant-based foods. They serve a vital role in impeding the absorption of dietary cholesterol, including the reabsorption of endogenous biliary cholesterol within the intestines, which is a crucial step in cholesterol reduction (Gupta *et al.*, 2011). As lipophilic phytochemicals, phytosterols constitute a significant portion of the unsaponifiable fraction found in plant oils (Ramadan *et al.*, 2006).

Avocado is known to contain the highest content of plant sterols among fleshy fruits (Duester, 2001). Codex Alimentarius Commission (2021) has proposed the sterol standards for avocado oil (Table 2.11). The sterol β-sitosterol stands out as the predominant sterol in avocado oil, followed by Δ-5-avenasterol, campesterol, and stigmasterol (Tan *et al.*, 2018a). Wong *et al.* (2010) have identified β-sitosterol as the primary phytosterol in cold-pressed avocado oil, ranging from 2.23 to 4.48 grams per kilogram oil. Meanwhile, Berasategi *et al.* (2012) reported the total phytosterol content in commercial avocado oil to be 3.39 g/kg.

Table 2.11: Sterols composition of avocado oil in CODEX proposed standards (Codex Alimentarius Commission, 2021)

Sterols	Proposed CODEX standards (% of total sterols)
Cholesterol	ND-0.5
Brassicasterol	ND-0.5
Campesterol	4.0-8.3
Stigmasterol	0.3-2.0
Clerosterol	0.6-2.0
Beta-sitosterol	71.0-93.4
Delta-5- avenasterol	2.0-8.0
Delta-7- stigmastenol	ND-3.5
Delta-7- avenasterol	ND-1.5

2.7 Conclusions

Avocado oil, especially extra-virgin cold-pressed avocado oil, is a highly desirable and premium edible oil known for its attractive appearance, gentle flavour and nutritional composition. The maturity of avocado fruits plays a crucial role for oil availability. Dry matter content serves as a reliable indicator of maturity and oil content, which shows a positive correlation. The ripeness of fruit is important for improving extraction efficiency during malaxing. The ripeness of fruits can be detected by skin colour changes and firmness assessment.

Avocado fruit comprises three main parts: the pulp (mesocarp), skin (exocarp), and seed (stone). Previous research mainly focused on pulp, which contains up to 30% oil when mature in that it is the main source of avocado oil. Avocado pulp contains bioactive phytochemicals such as polyphenols, carotenoids, and vitamins. The skin and seed are considered to be the by-product of avocado oil manufacturing, however they contain high phenolic content and antioxidants.

Mechanical aqueous extraction is the common method for avocado oil industry, which involves grinding avocado flesh, malaxing and centrifugation. In addition to traditional Soxhlet extraction, laboratory extraction of avocado oil can also be performed using Accelerated Solvent Extraction (ASE), Ultrasound-Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE), and the Supercritical CO_2 Method.

Traditional extraction methods involving heat may lead to undesired reactions, such as oxidation and lipid hydrolysis. Therefore, the determination of quality parameters for avocado oil is important, which involves PV, FFA, specific extinction coefficients, and oxidative stability. There is no globally standardized criteria for avocado oil so far, however CODEX has proposed a draft standard for avocado oil, which is currently under review.

The major chemical components in avocado oil, such as its fatty acid profile dominated by oleic acid, pigments like lutein and chlorophyll, phenolic compounds and phytosterols, contribute to its nutritional profile and potential health benefits. Bitterness determination in avocado oil still lacks a standardized method, which needs further sensory test and evaluation. Although current research has indicated that potential increase in the range and total phenolic content when the peels and seed are included, the utilization of avocado peel and seed in avocado oil is still an under-explored area with limited research. Further investigation is warranted to identify into the potential benefits or problems, and applications of incorporating avocado peel and seed in avocado oil extraction.

Chapter 3: Materials and Methods

3.1 Raw materials

3.1.1 Avocado fruit harvest

Early season is when avocados have reached minimum maturity and can be harvested for export, in New Zealand this is approximately July to August. Avocados can remain on the tree until the following year until April to July, fruit harvest then is considered late harvest. The harvest of 'Hass' avocado fruit was carried out at two time points, fruit harvested on the 22nd July 2022, was late season fruit from the previous season and fruit harvested on the 30th August 2022 was early season fruit for the new season, from Pat and Sue Tetley's orchard, Waiau Pa, Auckland. The avocados were delivered directly to the Mt. Albert Research Centre (MARC) of New Zealand Institute of Plant and Food Research Ltd in Auckland for the postharvest assessments and ripening. All malaxing and testing of oils extracted was carried out at the Massey University Auckland campus.

3.2 Postharvest assessments

3.2.1 Dry matter determination for avocado fruit

Avocado maturity was measured by determining dry matter content. Two plugs of avocado pulp were sampled from the equator using a Hofshi coring machine from a sample of 20 fruits. After removing the skin and seed coat, the prepared fruit plugs were sliced and the transverse slices weighing a total of between 2 to 3 grams were prepared. The avocado slices were placed in a petri dish and the exact weight of each of the fourty avocado cut plug tissues was measured to three decimal places. Subsequently, these avocado slices were dried in oven (Ezidri Ultra FD1000; Hydraflow Industries Ltd, Upper Hutt, New Zealand) maintained at a temperature of 65 ± 2 °C for 48 h, until a constant weight was achieved (Yang *et al.*, 2020). The dry matter content in the fruit was determined using Equation 3.1.

$$Dry\ matter\ (\%) = \frac{mass\ of\ dry\ flesh, g}{mass\ of\ wet\ flesh, g} \times 100 \qquad \text{Equation 3.1}$$

3.2.2 Ethylene Treatment and Ripening

After the determination of dry matter, the remainder of the unsampled fruit were put into single layer trays for storage and then underwent an ethylene treatment designed to accelerate the ripening process and reduce ripening variability between the fruit. The fruit were treated with 100 µL L^{-1} ethylene within a 360 L tub at controlled temperature of 20°C ± 2°C for a duration of 24 h. The tubs were taped to prevent ethylene leakage and the addition of ethylene was repeated twice at 12 h intervals, then the avocados were removed from the tubs and held at 20°C ± 2°C for a period of 5-8 days until ripe.

3.2.3 Firmness measurement for avocado fruit

During the ripening process after ethylene treatment, the firmness of fruit was measured every day to monitor the ripeness based on the hand firmness guide shown in Table 2.3. Once the fruit were fully ripe (Hand rating of 5), they could be processed for oil extraction or stored at 4°C for a maximum of one day before extraction.

3.3 Total oil content of avocado pulp, skin and seed by Soxhlet

3.3.1 Reagent

Hexane ≥ 97.0%: HPLC Grade, Sigma-Aldrich, USA

3.3.2 Preparation of sample

The pulp, peel and stone of avocado were separated, weighed and separately ground (Siemens grinder, Munich, Germany). Each type of tissue (flesh, skin, seed) was placed onto petri dishes and dried at 65 ± 1°C for approximately 48 h until they were at constant weight. These dried samples were ground into powder using a pestle and mortar, then placed into vacuum sealed foil bags at -20 ± 1°C for subsequent use.

3.3.3 Extraction of oil and calculation

Soxhlet extraction (Buchi, Switzerland) was used to extract the oils from different parts of the avocado fruit. Five grams of dried pulp (flesh) sample or 15 g of skin or seed samples were placed into cellulose thimble (33 × 94 mm, Buchi, Switzerland) and then the thimble was placed into the Soxhlet extractor. Ninety millilitres of hexane were placed into the 150 mL Soxhlet flask. The sample flask was positioned on the heating mantle. The extraction process was automated and ran for 6 h. The total oil collected from each sample was calculated using Equation 3.2

$$Total\ oil\ (g\ oil/g\ dry\ tissue) = \frac{(W_2 - W_1)}{P} \times 100\% \quad \text{Equation 3.2}$$

Where:

W_1 is the weight of flask (g);

W_2 is the weight of flask plus oil (g);

P is the weight of the dry sample (g).

3.4 Laboratory scale cold-pressed extraction of avocado oil

The extraction of cold-pressed avocado oil in a laboratory setting was conducted following the methodologies outlined in Figure 3.1, as described by Woolf *et al.* (2009), Wong *et al.* (2011) and Yang *et al.* (2019).

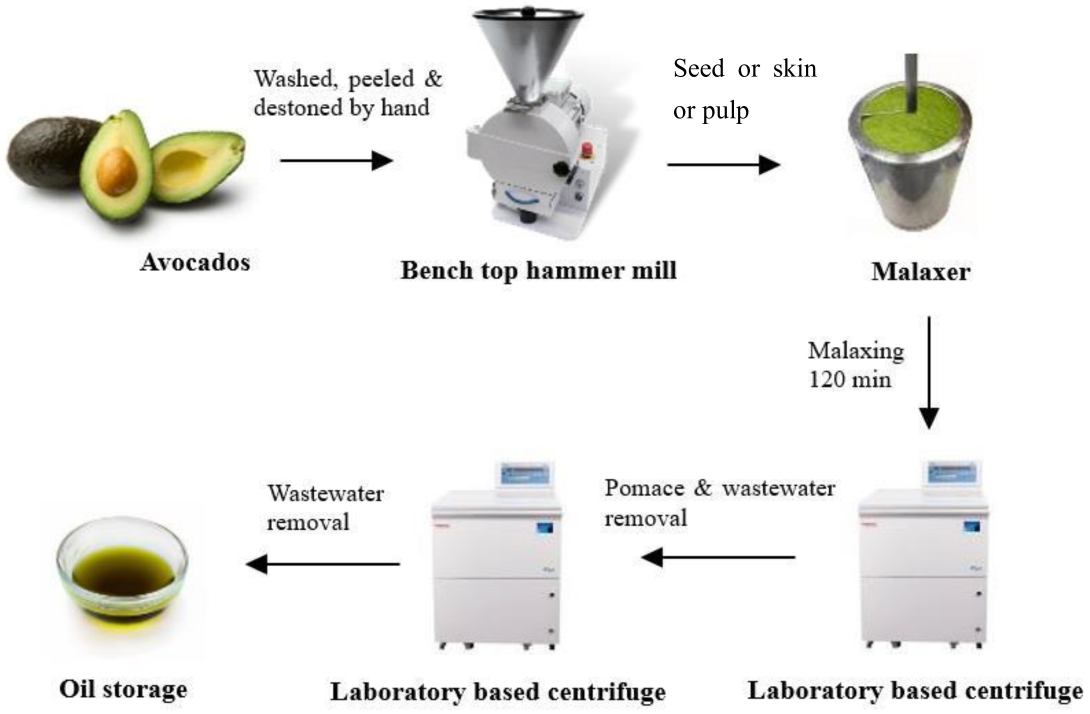

Figure 3.1. Flow chart of laboratory scale cold-pressed extraction of avocado oil (Yang, 2019)

3.4.1 Sample treatments

The samples were divided into three groups according to the treatments of fruit, which is shown in Table 3.1. As 90% skin is removed during the seed and skin removal stage in New Zealand cold-pressed systems, 10% of the skin was included in treatment A to simulate the standard amount during commercial malaxing (Costagli & Betti, 2015; Woolf *et al.*, 2009).

Table 3.1. Three different treatments of the avocado fruit – proportion (%) of available tissue type

Treatments	Flesh	Skin	Seed
Flesh only	100%	10%	0%
Flesh and skin	100%	100%	0%
Whole fruit	100%	100%	100%

3.4.2 Grinding

About six to eight avocado fruits were used for each of three replicate extractions. The fruit were first washed, cut into small pieces and then ground using a bench top hammer mill (Siemens, Germany), with a mesh size of 5 mm. Approximately 1 kg of the tissue was transferred into each stainless-steel vessel (malaxer) after grinding. Flesh and skin and/or seed were ground together.

3.4.3 Malaxing

The malaxing system was mainly composed of water bath and controller (Grant Instruments, USA), overhead stirrers and motors (Plant & Food Research Ltd, NZ) and 1 L stainless steel vessels (Bunnings Ltd, NZ) as shown in Figure 3.2. After the water bath temperature reached the target temperature (45 ± 1°C), the stainless-steel vessel with tissue was placed into the water bath with the overhead stirrer and frame. Each treatment was malaxed at 20 rpm for 90 minutes.

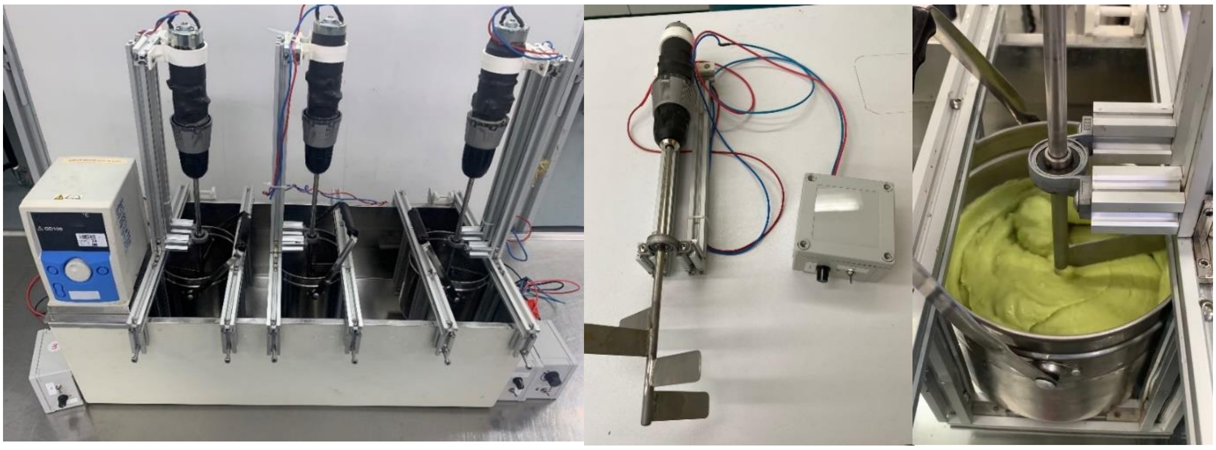

Figure 3.2. The water bath and malaxing system for cold pressed extraction of avocado oil

3.4.4 Centrifugation

Following the malaxing process, about 150 g of avocado pulp was placed into a 300 mL plastic centrifuge bottle. Subsequently, centrifugation was carried out at 12,278 *g* (8,500 rpm) (Sigma 6-16KS, Germany), for a duration of 30 minutes at a controlled temperature of 20 ± 1°C. This centrifugation step aimed to effectively separate the oil phase from both the solid residue (pomace) and the aqueous phase. The resulting oil phase was carefully collected using a pipette into a 30 mL plastic centrifuge tube and then recentrifuged again (Figure 3.3).

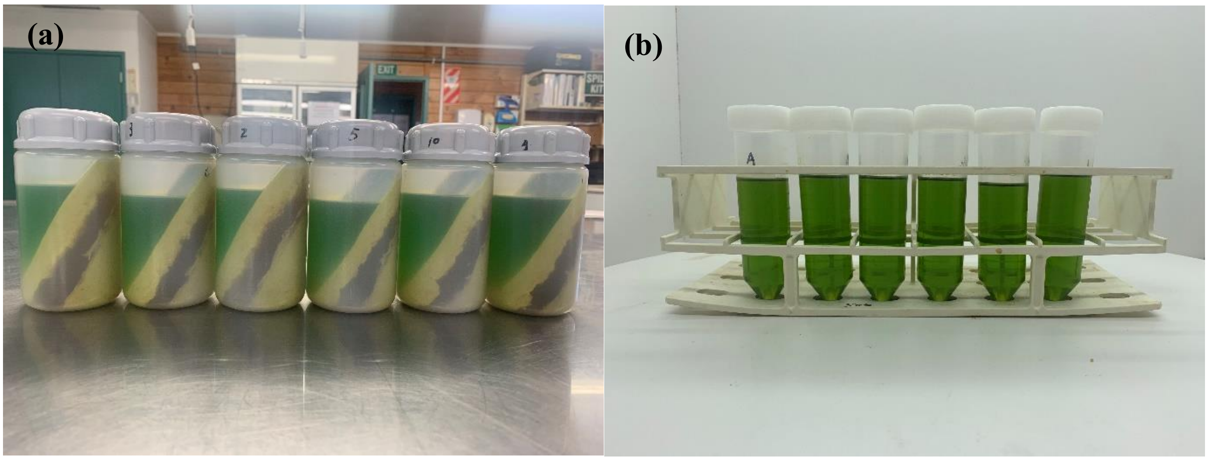

Figure 3.3. Avocado tissue after (a) first centrifugation and (b) second centrifugation

This second centrifugation was performed at 3,214 g (5,000 rpm) for 10 minutes, maintaining the temperature at 20 ± 1°C. The purpose of this additional centrifugation step was to ensure the elimination of any remaining traces of water from the oil phase. After collecting the upper oil phase into an amber glass bottle and the headspace of the bottle was flushed with nitrogen to remove oxygen, the samples were stored at -80 ± 2°C for further analysis.

3.4.5 Oil yield determination

Cold pressed oil yield after malaxing and centrifugation was calculated using Equation 3.3.

$$Oil\ yield\ (\%\ (\mathrm{g}\ oil/100g\ \text{wet tissue}\ in\ malaxer)) = \frac{W_{oil}}{W_{tissue}} \times 100\% \quad \text{Equation 3.3}$$

Where:

W_{oil} is the weight of oil collected after second centrifugation (g);

W_{tissue} is the weight of wet tissue in malaxer (g).

3.5 Storage trial

Avocado oil samples were carefully poured into 25 mL amber bottles in a dark environment and subjected to a process of nitrogen flushing to remove any oxygen from the headspace before being sealed. These oil-filled bottles were then labelled and stored under controlled conditions at various designated temperatures for the entire duration of the storage experiments. The specific storage temperatures employed were 20 ± 1 °C, 30 ± 1 °C, and 40 ± 1 °C in incubators (Qualtex, Andrew Thom Limited, Sydney, Australia, Model: 65R2). At each scheduled sampling day, one bottle of oil was removed from the incubators. The timeline of each treatment is shown in Table 3.2, where "√" indicated the day when oils were taken out from the incubator for testing.

Table 3.2. Timeline for the storage trial

Time (days)	Flesh only	Flesh and skin	Whole fruit
0			
13	√	√	√
27	√	√	√
41			√
55	√	√	√
83			√
97	√	√	
111			√
139	√	√	
167		√	
195	√		

√ - indicates the time when oil sample was tested

3.6 Avocado oil quality analysis

3.6.1 Peroxide value determination

The determination of PV was based on AOCS Method Cd 8b-90; Acetic Acid-Isooctane Method (AOCS, 2017).

3.6.1.1 Reagents

- Sodium thiosulphate pentahydrate ≥ 99.5%: ACS reagent, Sigma-Aldrich, USA
- Acetic acid ≥ 99%: glacial, ReagentPlus®, Sigma-Aldrich, USA
- Iso-octane ≥ 99.8%: HPLC grade, Fisher Scientific, USA
- Potassium iodate: AnalR®, BDH, United Kingdom
- Hydrochloric acid 32%: Analytical Reagent Grade, Fisher Scientific, United Kingdom
- Potassium iodide (iodate free) ≥ 99%: Analytical Reagent Grade, LabServ™, Thermo Fisher Scientific, New Zealand
- Sodium carbonate ≥ 99.8%: Anhydrous, Analytical Reagent, Univar, Ajax Finechem, Thermo Fisher Scientific, New Zealand

- Starch: soluble, ACS (for iodometry), Alfa Aesar, Thermo Fisher Scientific, USA

3.6.1.2 Preparation of reagents

0.1M Sodium thiosulphate: A 0.1M solution of sodium thiosulphate was prepared by dissolving 24.81 ± 0.01 g of sodium thiosulphate pentahydrate ($Na_2S_2O_3$) and 0.05 g of sodium carbonate in MilliQ water to make up a final volume of 1 L. The prepared solution was stored in a dark environment and standardized weekly for up to four weeks.

0.01M Sodium thiosulphate: A 0.01 M solution of sodium thiosulphate pentahydrate was prepared each time before use by diluting 10 mL of a 0.1M sodium thiosulphate pentahydrate solution with MilliQ water in a 100 mL volumetric flask.

Saturated Potassium Iodide (KI): A saturated KI solution was made by dissolving approximately 10 g of KI in 5 g of water. The solution was kept in a dark environment and tested before use. Saturated potassium iodide is prepared a day in advance and stored overnight. It was checked for undissolved crystals before use to ensure that the solution was saturated.

Starch indicator (1%): To make a 1% starch indicator solution, 1 g of soluble starch was first dissolved in 2 g of cold water and then made up to 100 mL with boiling water. The mixture was further boiled for one minute. The prepared solution was stored in the dark and discarded once the first sign of precipitate appeared.

Potassium iodate (KIO_3): The primary standard KIO_3 should be oven dried at 120°C for 1 hour or overnight before use, which was cooled in a desiccator after drying.

Acetic acid and iso-octane blend: Acetic acid and iso-octane are mixed in a 3:2 volumetric ratio and stored in a dark environment.

3.6.1.3 Standardisation of 0.1M sodium thiosulphate solution

The standardisation of 0.1M sodium thiosulphate solution was carried out weekly. The primary standard KIO_3 was accurately weighed out at 0.05 - 0.1 g plus approximately 2 g of KI (iodate free), both were dissolved in 75 mL MilliQ water in a 250 mL conical flask. Two millilitres of 6M hydrochloric acid (HCl) were added to the flask and was immediately titrated with 0.1M sodium thiosulphate solution until it turned a persistent pale-yellow colour. Half a millilitre of 1% starch indicator solution was added and the titration continued until the violet blue/black colour disappeared. The volume of 0.1M sodium thiosulphate solution was recorded for determining its molar concentration. A blank titration was carried out by replacing the sample with water.

The normality of the sodium thiosulphate solution was calculated using Equation 3.4.

$$N = \frac{(28.037 \times W)}{(S - B)} \qquad \text{Equation 3.4}$$

Where:

N = normality of sodium thiosulphate (mol L^{-1});

W = weight of KIO_3 (g);

S = volume (mL) of sodium thiosulphate titrated;

B = volume (mL) of blank.

3.6.1.4 Acetic Acid Isooctane Method for determining PV

Oil samples were first inverted to ensure a well-mixed sample. Five grams of oil was weighed in a 250 mL conical flask and dissolved into 50 mL acetic acid and iso-octane blend. Half a millilitre of saturated KI was added into the flask and reacted for about one minute. Throughout the reaction, the solution was shaken well at least three times. Subsequently, 30 mL of MilliQ water was added. The solution was immediately titrated with a 0.01M sodium thiosulphate pentahydrate solution until a persistent faint yellow colour was achieved. After that, 0.5 mL starch solution was added and the titration continued until the blue/black colour disappeared. The volume of sodium thiosulphate pentahydrate solution titrated was used to calculate the peroxide value of the oil sample.

The blank titration was completed by only using acetic acid isooctane blend before determination for omitting the influence of solvent.

The determination of peroxide value can be calculated with Equation 3.5

$$PV = [(S - B) \times N \times 1000]/W \qquad \text{Equation 3.5}$$

Where S = volume (mL) of sodium thiosulphate titrated

B = volume (mL) of blank

N = calculated normality of std thiosulfate solution

W = weight of oil sample (g)

PV = meq active oxygen/kg sample (meq peroxide/kg oil)

3.6.2 Free fatty acid (FFA) determination

The determination of FFA was based on AOCS Official Method Ca5a-40 (AOCS, 2017).

3.6.2.1 Reagents

- Phenolphthalein solution: 1% in methylated spirit, Fisher Scientific
- Sodium hydroxide (NaOH), pellets ≥ 97.0%: Analytical reagent, Univar, Thermo Fisher Scientific, New Zealand
- Potassium hydrogen phthalate (KHP) ≥ 99.96%: Analytical reagent grade, Fisher Scientific, United Kingdom
- Ethanol absolute: LabServ Analytical, Reagent Grade, New Zealand.

3.6.2.2 Preparation of reagents

Phenolphthalein indicator (1%): Phenolphthalein dye (1 g) was dissolved in 100 mL absolute ethanol.

0.1M Sodium hydroxide solution: Four grams of NaOH pellets were dissolved and made up to 1 L in a volumetric flask with MilliQ water.

Potassium hydrogen phthalate (KHP): KHP was dried at 120 ± 0.5°C in oven for 2 hours and cooled in a desiccator.

3.6.2.3 Standardisation of KHP

In order to standardize the sodium hydroxide solution, 0.2 g of dried potassium hydrogen phthalate was dissolved in 25 mL MilliQ water in a 100 mL conical flask. The flask was gently heated on a hot plate to aid in dissolution. Once the solution was cooled, three drops of 1% phenolphthalein indicator were added. The NaOH solution was then titrated into the flask until the first faint pink colour remained for 30 seconds. The volume of NaOH solution used in the titration was recorded and utilized for calculating the normality of the sodium hydroxide using Equation 3.6.

$$N = \frac{g\ of\ KHP}{ml\ of\ NaOH \times 0.204} \qquad \text{Equation 3.6}$$

3.6.2.4 Determination of %free fatty acid

To determine the %free fatty acid (FFA) content of the oils, a 1 g sample of the oil was accurately weighed to three decimal places and placed into a 100 mL conical flask. Subsequently, 20 mL of absolute ethanol was neutralized to the endpoint with the addition of a few drops of phenolphthalein indicator and titrated using 0.1M NaOH until a permanent faint pink colour emerged. After neutralization, the ethanol was gently heated to 50 ± 1°C and poured into a conical flask containing the oil sample to dissolve it. The dissolved sample was subjected to titration using 0.1M ethanolic NaOH, with the flask being gently shaken during this process. The titration process persisted until the initial permanent pink colour appeared, which should match the intensity of the neutralized solvent's colour before its addition to the sample. The percentage of FFA was calculated based on the Equation 3.7 based on oleic acid equivalent.

$$\%FFA\ as\ oleic\ acid\ w/w = \frac{mL\ of\ NaOH \times N \times 28.2}{Weight\ of\ sample, g} \qquad \text{Equation 3.7}$$

Where N = normality of NaOH, mol/L.

3.6.3 Specific extinction coefficient determination (K_{232}, K_{270})

The determination of specific extinction coefficient was based on the International Olive Council Method T.20/Doc. No 19/Rev.5 (International Olive Council, 2019a).

3.6.3.1 Reagents

- Cyclohexane≥99.0%: Analytical reagent, Univar, Asia Pacific Specialty Chemicals Ltd, New Zealand

3.6.3.2 Determination of K_{232} and K_{270}

An accurately weighed sample of oil (0.25 g) was placed into a 25 mL volumetric flask which as then filled to the mark with cyclohexane. The solution was carefully swirled to ensure homogeneity, otherwise it was filtered until no suspended impurities are observed by 0.22 µm nylon syringe filter. The clear solution was put in quartz cuvette to measure the absorbance at UV 266, 270 and 274 nm. To measure the absorbance at UV 232 nm, a second accurately weighed sample of oil (0.05 g) was added to a 25 mL volumetric flask and filled to the volume with cyclohexane.

The specific extinctions determined at 232 nm and 270 nm were calculated using Equation 3.8.

$$K\lambda = \frac{E\lambda}{c \times s} \qquad \text{Equation 3.8}$$

Where:

Kλ is the specific extinction at wavelength;

c is the concentration of the solution in g/100 mL;

s is the path length of the quartz cell in cm.

3.6.4 Total phenolics determination

The determination of total phenolics was based on the Folin-Ciocalteu method reported by Singleton *et al.* (1999)and Vinson *et al.* (2005).

3.6.4.1 Reagents

- Folin- Ciocalteu phenol reagent (FCR) 2N: Sigma-Aldrich, USA
- Caffeic acid ≥ 98.0%: HPLC grade, Sigma-Aldrich, USA
- Methanol ≥ 99.9%: LC-MD Grade, Fisher Scientific, United Kingdom
- Hexane ≥ 97.0%: HPLC Grade, Sigma-Aldrich, USA

- Sodium carbonate ≥ 99.8%: Anhydrous, Analytical Reagent, Univar, Ajax Finechem, Thermo Fisher Scientific, New Zealand
- Formic acid ≥ 98%: ACS reagent, EMSURE®, Merck, Germany
- Ethanol absolute: LabServ Analytical, Reagent Grade, New Zealand.

3.6.4.2 Preparation of reagents

Acidified water (pH 2.5): To prepare, formic acid was gradually added to 10 mL MilliQ water to adjust the pH to 2.5 ± 0.5.

Methanol and acidified water mixture (80:20): Ten millilitres of acidified water were added into a 50 mL volumetric flask and made up to the mark with methanol. After swirling for a minute, more methanol was added to make up the volume if the liquid level went down.

Folin-Ciocalteu solution (FCR): Five millilitres of FCR were added to a 50 mL volumetric flask and made up to the mark with MilliQ water.

Caffeic acid stock solution: A 0.5mg/mL caffeic acid solution was prepared by dissolving 125 mg caffeic acid in absolute methanol made up to 250 mL.

Sodium carbonate solution (75 g/L): Sodium carbonate (3.75g) was dissolved in MilliQ water in a 50 mL volumetric flask and made up the volume.

3.6.4.3 Standard curve determination

To prepare a standard curve, different volumes of caffeic acid stock solution and methanol were added into 20 mL Kimax text tubes, according to Table 3.3.

Table 3.3 Composition ratio of standard curve solution

Stock solution	Volume added to test tube of stock solution	Volume of methanol to make up to 1mL	Concentration of caffeic acid (mg mL^{-1})
0.5 mg/mL	0	1.0	0.0
	0.05	0.95	0.025
	0.075	0.925	0.0375
	0.1	0.90	0.05
	0.125	0.875	0.0625
	0.15	0.85	0.075
	0.2	0.8	0.10
	0.25	0.75	0.125

To each test tube containing 1 mL of diluted sample, 5 mL of FCR was first added. Then 4 mL sodium carbonate solution was added after 3-8 minutes. The test tubes were sealed with the lids and incubated in a dark environment at room temperature for 90 min. The zero mg/mL sample was used as the blank in the spectrophotometer (Shimadzu UV-1700, Kyoto, Japan). The absorbance of each sample in quartz cuvettes was read at a wavelength of 760 nm. The standard curve was obtained by plotting the caffeic acid concentration on the x- axis and absorbance at 760 nm on the y- axis.

3.6.4.4 Phenolic compound extraction

Before the Folin-Ciocalteu test, the phenolics were extracted into methanol from the oil. In the extraction process, approximately 10 mL of oil was weighed and mixed with 10 mL of hexane in a beaker. The mixture of oil and hexane was then transferred into a 100 mL separation funnel. The beaker was rinsed twice with 5 mL of hexane, which was also collected in separation funnel. To extract phenolic compounds, 30 mL of a methanol-acidified water mixture was added to the separating funnel and the contents were vigorously shaken for two minutes. During the shaking process, the lid of funnel was intermittently opened to avoid excessive pressure build up. The water-based layer that settled at the lower portion of the separation funnel was collected in a 250 mL round-bottom flask. The extraction procedure was repeated twice more, employing an extra 30 mL of methanol-acidified water in each repetition. The aqueous layers from these subsequent extractions were combined in the same 250 mL round-bottomed flask. The mixed aqueous fraction was subjected to drying by a rotary evaporator (Buchi, Switzerland). The round-bottomed flask containing the aqueous solution was placed in a water bath set at 40 ± 1°C to facilitate the evaporation process. The residue was dissolved in 3 mL of absolute methanol and thoroughly mixed. The resulting sample solution was carefully pipetted into a small sample vial and stored at -20°C until further analysis.

3.6.4.5 Determination of total phenolics

The extracted sample of phenolics prepared in Section 3.6.4.4 was diluted to 10 mL in a falcon tube with absolute methanol. The dilution amount was recorded e.g. if 3 mL extract was put into falcon tube and made up to 10 mL then the dilution factor was 10/3=3.33. Total phenolic reaction was carried out as shown in Table 3.4. The reaction could also be carried out using 0.2 mL diluted extraction sample if the sample was not sufficient.

Table 3.4. The volume needed for each reagent in total phenolic reaction

Sample (mL)	Folic Ciocalteau (mL)	Sodium Carbonate (mL)	Total volume (mL)
1	5	4	10
0.2	1	0.8	2

The specific experimental steps are the same as the preparation of the standard curve (Section 3.6.4.3). The total phenolics content was calculated based on the standard curve mg caffeic acid/mL MeOH (Equation 3.9).

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

Equation 3.9

The outcome was expressed in milligrams of phenolics per kilogram oil as caffeic acid equivalents.

3.6.5 Colour determination

The colour of the oil was assessed using the Minolta CR300 colorimeter (Minolta, Japan). Prior to conducting measurements on the oil samples, the colorimeter was calibrated using a standard white tile. The oil was placed in 4 mL plastic cuvettes with an optical pathway of 10×10 mm. Each cuvette was sequentially positioned in the liquid sample holder, and colour measurements were performed. For each replicate, three measurements were taken, and the average reading was recorded.

3.6.6 Total chlorophyll determination

Pheophytin represents the predominant chlorophyll pigment present in unprocessed vegetable oils. The total chlorophyll content in vegetable oil was expressed in milligrams of pheophytin *a* per kilogram of oil, in accordance with the AOCS Ch 4-91 methodology (AOCS, 2017).

For the analysis of total chlorophyll concentration, a division constant of 0.101 was utilized, which is a factor associated with the spectrophotometer's performance as applied in the analysis of virgin avocado oil (Sherpa, 2002). The determination of total chlorophyll was conducted in accordance with AOCS Ch 4-91 with certain modifications as outlined in AOCS (2017) and Sherpa (2002)

The procedure entailed the dilution of avocado oil, with a mixture of two millilitres of avocado oil and three millilitres of isooctane. Subsequently, the absorbance was measured using a UV-Visible Spectrophotometer (UV-1700 PC, Shimadzu). Measurements were taken at wavelengths of 630 nm, 670 nm, and 710 nm. The resulting absorbance values were recorded. The calculation of the total chlorophyll concentration was carried out using Equation 3.10.

$$C_{670} = \frac{A_{670} - 0.5(A_{630} - A_{710})}{0.101 \times L \times D} \qquad \text{Equation 3.10}$$

Where:

C670 = Total chlorophyll pigment content (ppm);

A_n = Absorbance of oil at wavelength n;

L = Path Length (1 cm);

D = Dilution = 0.4.

3.6.7 Determination of the degradation products of chlorophylls *a* and *a'* (pheophytins *a, a'* and pyropheophytins)

The determination method for pyropheophytin was based on an ISO method (ISO (2009), the peak identification of the chromatogram was based on ISO 29841 and Gómez-Coca *et al.* (2020).

3.6.7.1 Reagents

- Chlorophyll *a* standard: Analytical standard, Sigma-Aldrich, USA
- Acetone: Analytical reagent, Thermo Fisher Scientific, New Zealand
- Methanol ≥ 99.9%: LC-MD Grade, Fisher Scientific, United Kingdom
- Heptane: HPLC grade, Prolabo, VWRTM, New Zealand
- Silica gel 60 (0.063-0.100 mm): Merck, Germany
- Petroleum ether (boiling point range 40°C to 60°C): Analytical reagent grade, Fisher Scientific, United Kingdom
- Diethyl ether: Analytical reagent grade, Lab Serv$^{\text{TM}}$, New Zealand

3.6.7.2 Preparation of reagents

Silica gel column: The silica gel was first dried in an oven at 50 ± 1°C and hydrated with MilliQ water to achieve 5% hydration. A small plug of cotton wool, measuring around 5 mm in height, was introduced into the bottom section of a 5 mL pipette tip. This was followed by the addition of 500 mg of 5% hydrated silica gel 60. A further plug of cotton wool, also approximately 5 mm high, was positioned on top of silica gel layer. Gentle compression of the contents was achieved by gently pressing down on the upper cotton wool using the flat tip of a stirring rod.

HPLC Mobile phase: MilliQ water was blended with acetone and methanol to a volumetric ratio of 1:9:15 water:methanol:acetone. This mobile phase was filtered using 0.22 µm nylon filters (Millipore Durapore membrane filter, 0.22 µm GV, USA) and was degassed prior to utilization.

Petroleum ether and diethyl ether solvent mixture: Petroleum ether was combined with diethyl ether in a volumetric ratio of 9:1.

3.6.7.3 High Performance Liquid Chromatography (HPLC) chromatographic conditions

The HPLC used was Shimadzu-10A system (Shimadzu, Japan). The column used was a Phenomenex Luna C18(2) (5 µm 150 x 4.60 mm i.d). A sample volume of 10 µL was injected, the mobile phase flow rate was 1.0 mL/min, while maintaining the column temperature at 30 ± 1°C. All mobile phases were first filtered using 0.22 µm nylon filters (Millipore Durapore membrane filter, 0.22 µm GV, USA). Detection was performed by UV detection at a wavelength of 410 nm using a Shimadzu SPD-20A UV/VIS detector.

3.6.7.4 Identification and separation of pheophytin a, pheophytin a' and pyropheophytin

Elevated temperatures can induce the breakdown of chlorophyll, resulting in the detachment of the magnesium ion (van Boekel, 2000). Additionally, the formation of pyropheophytin occurs due to the removal of the carbomethoxy group. To assess the impact of chlorophyll degradation, a commercial avocado oil sample was heated at 100°C for 60mins. This approach aimed to discern distinctive peaks corresponding to pheophytin *a*, pheophytin *a',* and pyropheophytin by comparing the peak changes before and after heating and also with the peak shape and retention time in the reference (Gómez-Coca *et al.*, 2020; ISO, 2009)

3.6.7.5 Extraction of chlorophyll pigments

A 300 mg oil sample was accurately weighed and placed in a small glass beaker, where it was dissolved in 1 mL of heptane. It was then carefully transferred onto a silica gel 60 cartridge. The beaker was rinsed twice using 1 mL petroleum ether, and washings also being directed onto the cartridge. Once the solvent had fully drained from the upper cotton section of the cartridge, the non-polar components were eluted with 5 mL petroleum ether/diethyl ether solvent mixture, twice. The pheophytin fraction present on the silica gel was then eluted twice using 5 mL of acetone, which was then collected in a pear-shaped flask, protected against exposure to light. The eluted sample along with the solvent was evaporated to dryness using a rotary evaporator (Buchi, Switzerland) at 20 ± 1°C. The sample after evaporation was reconstituted in 800 µL of acetone. This solution was immediately injected into the HPLC. It was important to note that no samples were retained for more than 30 minutes before being injected into the HPLC system.

3.6.7.6 Analysis of results

Pheophytin a, pheophytin *a'*, and pyropheophytin were distinguished based on their specific retention times and the areas under their respective peaks (Figure 3.4).

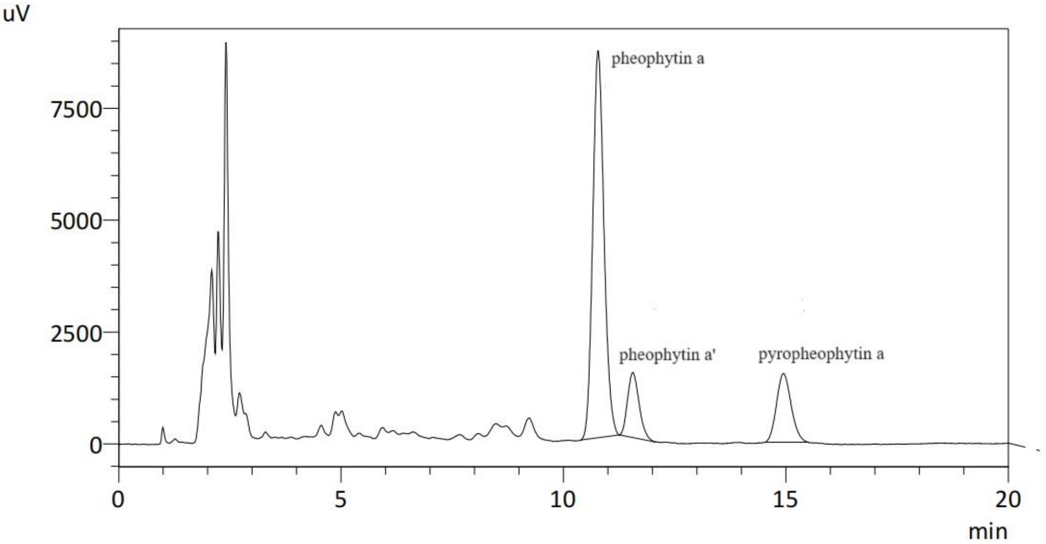

Figure 3.4 High-performance liquid chromatogram (HPLC) chromatogram of chlorophyll pigments of avocado oil at 410nm

These peak areas were used to calculate the relative concentrations of these compounds within the sample solution, following the guidelines outlined in ISO (2009) and Gómez-Coca *et al.* (2020). The results are presented as a percentage mass fraction of pyropheophytin *a* using equation 3.11.

$$\omega_{PPPa} = \frac{A_{PPPa} \times 100}{(A_{PPPa} + \sum A_{pp})} \qquad \text{Equation 3.11}$$

Where:

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

$\sum A_{PP}$ is the sum of the peak areas of pheophytin *a* and *a'*.

3.6.8 Bitterness Index determination (K_{225})

The determination of bitterness index was reported by Ran (2014).

3.6.8.1 Reagents

- Methanol ≥ 99.9%: LC-MD Grade, Fisher Scientific, United Kingdom
- n-Hexane ≥ 97.0%: HPLC Grade, Sigma-Aldrich, USA

3.6.8.2 Extraction of phenolics

Avocado oil, 0.25g accurately weighed, was dissolved in 5 mL of n-hexane. To this solution, a 5 mL mixture of methanol and MilliQ water (60:40 v/v) was added. The resulting mixture was vigorously mixed using a vortex for a duration of two minutes and then subjected to centrifugation at 2470 *g* for a period of 10 min. Following centrifugation, the upper hexane layer was carefully separated, leaving behind the polar fraction. This polar fraction was carefully transferred into a 25 mL volumetric flask. The extraction process was repeated three times to ensure thorough extraction. The polar fractions from the three repetitions were combined into one single 25 mL volumetric flask. The combined fraction was subsequently increased to a total volume of 50 mL using the MeOH/H_2O (60:40 v/v) mixture. The absorbance of this fraction was measured at wavelength of 225 nm using a spectrophotometer (Shimadzu UV-1700, Kyoto, Japan).

3.6.8.3 Calculation of bitter index (K_{225})

The bitter index K_{225} can be calculated using Equation 3.12.

$$K_{225} = \frac{A_s \times 1g}{W_s \times D} \quad \text{Equation 3.12}$$

Where:

A_s is the absorbance at 225 nm;

W_s is the weight of avocado oil in grams;

D is the dilution factor.

The intensity of bitterness was calculated by Equation 3.13 (Gutiérrez Rosales *et al.*, 1992):

$$IB = 13.33K_{225} - 0.837 \quad \text{Equation 3.13}$$

3.6.9 Rancimat analysis for oxidative stability determination

The Rancimat method serves as an accelerated aging assessment, involving the bubbling of air through the sample contained within a reaction vessel, held at a consistently heightened temperature, which is based on AOCS Cd 12b-92 and ISO 6886 (AOCS, 2017; ISO, 2016). During this procedure, the fatty acids within the sample undergo oxidation. As the test concludes, volatile secondary byproducts of these reactions emerge and are carried into the measurement vessel by the airflow, subsequently being soaked up by the measuring solution (MilliQ water). Through this process, the ongoing electrical conductivity of the measuring solution steadily rises, attributable to the absorption of the resultant reaction byproducts. The period preceding the identification of secondary reaction compounds is referred to as the "induction time", which characterizes the oxidation stability of the fats and oils (Hamilton & Allen, 1994)

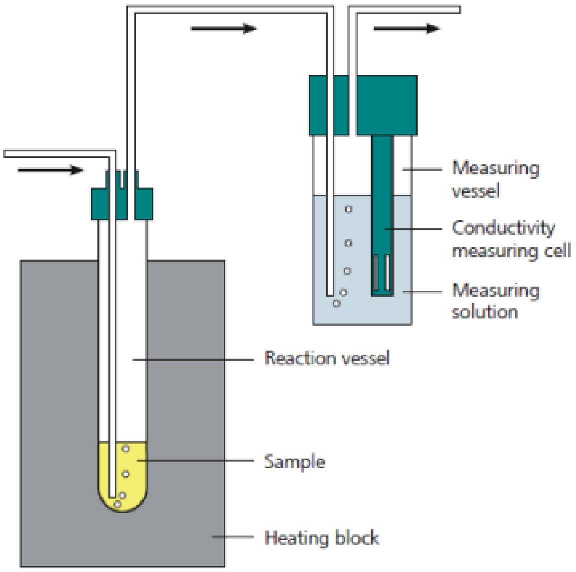

Figure 3.5 Rancimat process diagram (Metrohm, 2019)

The 892 Professional Rancimat (Metrohm & Co) was used to measure the induction time for the avocado oil samples (Figure 3.5). Three grams of avocado oil was directly weighed into each reaction vessel. The vessels were then placed into the heating block, heated to 110°C with an air flow rate of 20 L/h, the temperature correction was set to 1.6°C. Each sample was tested in duplicate and the results were quantified in terms of hours.

3.7 Avocado oil composition analysis

3.7.1 Fatty acid profile

The gas chromatography method for determining fatty acid composition of avocado oil was based on the Hartman and Lago (1973) and reported by Rwothomio (2011).

3.7.1.1 Reagents

- Methanol ≥ 99.9%: LC-MD Grade, Fisher Scientific, United Kingdom
- Acetonitrile ≥99.95%: HPLC Grade, Fisher Scientific, United Kingdom
- Potassium hydroxide ≥ 86.2%: Analytical reagent grade, Fisher Scientific, United Kingdom

- Concentrated sulfuric acid (98%): Analytical reagent, Univar, Thermo Fisher Scientific, New Zealand
- Ammonium chloride ≥ 99.5%: Analytical Grade, LabServ^{TM}, Thermo Fisher Scientific, New Zealand

3.7.1.2 Reagents preparation

Hartman reagent: For the preparation of the Hartman reagent, 2 g of ammonium chloride was dissolved in 60 mL of absolute methanol within a round-bottom flask. Following this, 3 mL of concentrated sulfuric acid was introduced, and the mixture was subjected to reflux for a duration of 15 mins.

0.5M Methanolic KOH: KOH (2.8 g) was dissolved into 100 mL absolute methanol volumetrically.

3.7.1.3 Saponification and methylation of fatty acids

The saponification and methylation process for converting fatty acids into fatty acid methyl esters (FAME) followed the protocol outlined by Hartman and Lago (1973). A sample of avocado oil accurately weighed (0.1 ± 0.05 g) was placed into a Kimax test tube. To this oil sample, 0.5 mL of 0.5M methanolic potassium hydroxide solution was added, and the tube was then placed in a water bath (Grant, United Kingdom) set at 70 ± 0.5°C for a duration of 10 minutes. Hartman reagent (5 mL) was then added to the test tube and the heating continued for an additional 10 minutes at 70 ± 0.5°C. The extract was then separated by first adding 2 mL of hexane and 5 mL of MilliQ water. Thorough mixing of the solution was carried out to facilitate the separation of the fatty acid methyl esters into the hexane phase. A sample of the top layer (hexane layer) was placed into a 2 mL vial. A small quantity (0.5 µL) of the resulting supernatant layer was injected into the gas chromatography (GC) system (Shimadzu GC-2030) for analysis.

3.7.1.4 GC operating conditions

The gas chromatograph used was the Shimadzu GC-2030 (Shimadzu, Japan). The system included auto-injector AOC-20i, and an auto-sampler AOC-20s. The analytical column employed was a DB-wax capillary column (Agilent Technologies, USA) with dimensions of 30 meters in length, 0.32 mm in internal diameter, and a film thickness of 0.25 microns. The column temperature was maintained at 200°C, while the temperature for the flame ionization detector (FID) was set to 300°C. The injection port was set at 250°C. The operational parameters included a total pressure of 60 kPa, a purge pressure of 100 kPa, and an overall purge flow of 0.8 mL/min. The analysis was carried out in a split injection mode (10:1), utilizing nitrogen gas as the carrier with a total flow rate of 20 mL/min. The analysis duration for each individual sample was 50 minutes. Peaks were identified by external reference standards and results for each fatty acid identified were reported as percentages of the total fatty acids present.

3.7.2 Individual phenolics

The HPLC method of individual phenolics was reported by Rwothomio (2011).

3.7.2.1 Reagents

- Methanol ≥ 99.9%: LC-MD Grade, Fisher Scientific, United Kingdom
- Hexane ≥ 97.0%: HPLC Grade, Sigma-Aldrich, USA
- Acetonitrile ≥99.95%: HPLC Grade, Fisher Scientific, United Kingdom
- Acetic acid ≥ 99%: glacial, ReagentPlus®, Sigma-Aldrich, USA

3.7.2.2 HPLC chromatographic conditions

For quantification of individual phenolics a Synergi Fusion-RP 80 C18 column (4 um 250 × 4.60mm id) was used. Separation was performed using a gradient elution with a mobile phase consisting of water:acetic acid (99:1 v/v) designated as solvent A and acetonitrile as solvent B following the method outlined by Benavente-Garcıa *et al.* (2000). All mobile phases were first filtered using 0.22 µm nylon filters (Millipore Durapore membrane filter, 0.22 µm GV, USA) and were degassed prior to utilization. Acetic acid was incorporated to maintain a consistent acid concentration throughout the gradient runs. The mobile phase's acidic pH served to prevent the dissociation of phenolic compounds, while also enhancing peak symmetry and reducing peak tailing, as noted by Harwood and Aparicio (2000). The specific elution gradient used is detailed in Table 3.5. The flow rate was set at 0.8 mL/min and the column temperature was maintained at 30 ± 0.5°C. The analysis was carried out by utilizing UV detection at 280 nm with a Shimadzu SPD-M20A diode array detector.

Table 3.5 The elution gradient for individual phenolic separation by HPLC

Time(mins)	Solvent A (%) Water/acetic acid (99:1 v/v)	Solvent B (%) Acetonitrile
0	95	5
20	75	25
40	50	50
50	20	80
60	95	5

3.7.2.3 Reference phenolics standards

The reference phenolic standards used for identification of individual phenolics are listed in Table 3.6.

Table 3.6 The commercial grade phenolic standards

Compound name % purity specification	Grade, Supplier
Benzoic acid ≥99.5%	ACS reagent, Sigma-Aldrich
Gentistic acid ≥98% (2,5-dihydroxybenzoic acid)	HPLC, Aldrich
p-coumaric acid (4-hydroxycinnamic acid)	HPLC, Sigma
Vanillic acid (4-hydroxy-3-methoxybenzoic acid)≥97%	HPLC, FlUnited Kingdoma
Caffeic acid≥98%	HPLC, Sigma-Aldrich
Sinapinic acid ≥99%	HPLC, Aldrich
Ferulic acid ≥99%	HPLC, Aldrich
Rutin ≥97%	HPLC, Acros
Quercetin ≥95%	HPLC, Sigma
Epicatechin≥90%	HPLC, Sigma

Kaempferol≥90%	HPLC, Sigma
Trans-Cinnamic acid≥99%	HPLC, Aldrich

3.7.2.4 Extraction of phenolics compounds

Before analysis of oils for individual phenolic identification the phenolics were extracted from the oil into methanol. Avocado oil (2.5 g) was accurately weighed into a centrifuge tube. Five millilitres of MeOH:H_2O (80:20 v/v) mixture was added to the oil and vortexed for 2 mins. After centrifugation at 2470 *g* for 10 minutes, the bottom layer was collected and the extraction process was repeated twice using a 5 mL of MeOH:H2O mixture for each repetition. The collected extracts were combined and concentrated under vacuum at temperatures below 35°C until nearly dry. For purification, 0.5 mL of acetonitrile and 2 mL of hexane were added to the concentrated phenolic extract (Tovar *et al.*, 2001). The resulting solution was then evaporated under vacuum to dryness. Finally, the phenolic extract was dissolved in 1 mL of methanol, which was then transferred to a 2 mL vial. From this solution, an aliquot of 10 µL was injected into the HPLC system for analysis.

3.7.2.5 Results analysis of individual phenolic

The identification of phenolic compounds was accomplished by correlating their UV spectra and retention times with those of external reference standards, as presented in Table 3.6. All phenolics were identified at a wavelength of 280 nm (Figure 3.6). The quantification of the identified phenolic compounds was determined using a regression equation derived from the relationship between peak areas and known concentrations of the external reference standards obtained from the same batch. Consequently, the concentrations of known phenolic compounds within the oil were expressed in terms of milligrams per kilogram of oil, while the unknown phenolics were expressed as milligrams per kilogram of oil on caffeic acid equivalent.

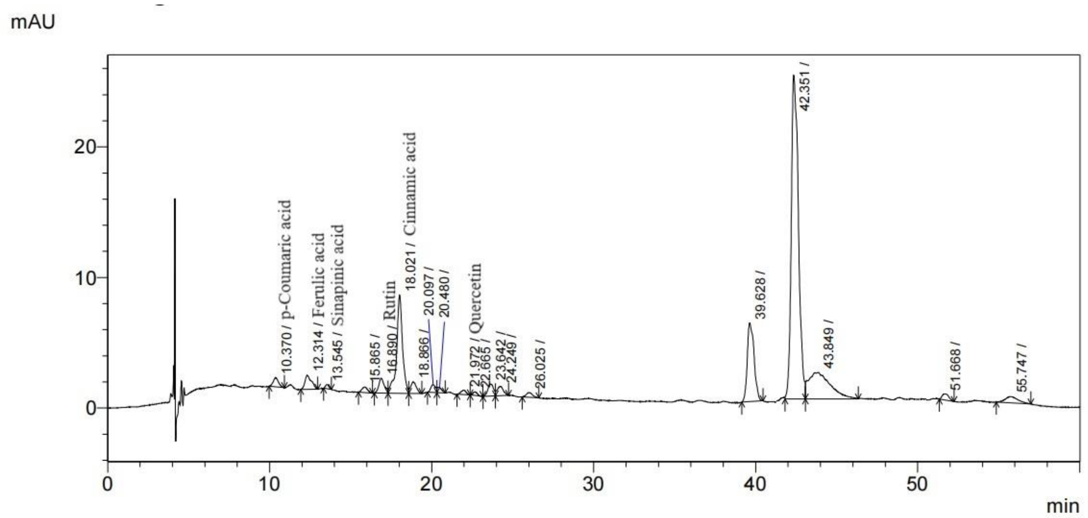

Figure 3.6 High-performance liquid chromatogram (HPLC) chromatogram of phenolic compounds of avocado oil at 280nm

3.7.3 Carotenoids and Chlorophylls

The determination of carotenoids and chlorophylls was based on the solid-phase extraction method proposed by Minguez-Mosquera *et al.* (1992) and Mateos and García-Mesa (2006).

3.7.3.1 Reagents

- Methanol ≥ 99.9%: LC-MD Grade, Fisher Scientific, United Kingdom
- Hexane ≥ 97.0%: HPLC Grade, Sigma-Aldrich, USA
- Acetone: Analytical reagent, Thermo Fisher Scientific, New Zealand
- Ammonium acetate: ≥97%, analytical reagent, Univar, Ajax Finechem, Thermo Fisher Scientific, New Zealand
- Butylated hydroxytoluene (BHT) ≥ 99%: SAFC, Sigma-Aldrich, Germany
- Triethylamine: HPLC grade, Fisher Scientific, United Kingdom
- Chlorophyll *a* standard: Analytical standard, Sigma-Aldrich, USA
- Chlorophyll *b* standard: Analytical standard, Sigma-Aldrich, USA
- Lutein standard: Extrasynthese, France

3.7.3.2 Sample preparation and extraction

Solid-phase extraction (SPE) was utilized for the isolation of chlorophylls and carotenoids from the oil sample. The SPE cartridge (Bond Elut Diol, 500 mg, 10 mL, Agilent, New Zealand) was initially conditioned by passing 8 mL of acetone followed by 8 mL of hexane. Approximately 0.8 g of avocado oil (accurately weighed) was mixed with 4 mL of hexane, containing 1% BHT (butylated hydroxytoluene), and then applied to the prepared SPE cartridge. After a first washing step with 4 mL of hexane, the elution of lutein and chlorophyll was conducted by washing twice with 4 mL of acetone, and the eluate was collected. The first hexane eluted fractions were all collected for the β-carotene determination. The lutein and chlorophyll eluates in acetone were evaporated to dryness in the rotary evaporator (Buchi, Switzerland) at 20 ± 1°C and then redissolved in 2 mL acetone. This was then stored in a 2 mL vial to HPLC injection.

3.7.3.3 β-carotene Determination

The entire hexane fraction was carefully collected and placed into a 10 mL volumetric flask, which was then filled up to the 10 mL mark with hexane. The resulting hexane phase contained the β-carotene fraction, which was directly quantified within this solution (after adjusting the volume to 10 mL) using its specific extinction coefficients in hexane: $\mathrm{E}_{450}^{1\%} = 2592\ (\beta - \mathrm{carotene})$ (Mateos & García-Mesa, 2006; Wrolstad *et al.*, 2004).

$$C_{\beta-carotene} = \frac{A \times V_1}{A^{1\%}} \times C^{1\%} \qquad \text{Equation 3.14}$$

Where:

A = absorbance of the hexane solution;

V_1 = dilution factor;

$A^{1\%}$ = the absorbance of a 1% solution (the extinction coefficient 2592 AU);

$C^{1\%}$ = the concentration of a 1% β-carotene solution (10mg/mL).

3.7.3.3 HPLC chromatographic condition for carotenoids and chlorophylls

The SphereClone 5μm ODS (2) 80Å LC Column (Phenomenex, 250 × 4.6 mm i.d.)

was used for separating the pigments. A Shimadzu-20A HPLC equipped with SPD-M20A diode array detector (Shimadzu, Japan) was used. The mobile phase flow rate was 1.0 mL/min at room temperature. The mobile phases consisted of a mixture of methanol and water (8:2 v/v) containing 0.025% ammonium acetate and 0.05% triethylamine as solvent A, along with a mixture of methanol and acetone (1:1 v/v) as solvent B. The chromatographic separation was achieved utilizing a solvent gradient as outlined in Table 3.7. All mobile phases were first filtered using 0.22 µm nylon filters (Millipore Durapore membrane filter, 0.22 µm GV, USA).

Table 3.7. Solvent programme for HPLC determination of carotenoids and chlorophyll

Time (min)	%A	%B
Start	75	50
10	50	50
12.5	50	50
14.0	20	80
16.0	20	80
21.0	0	100
35.0	0	100
40.0	75	25

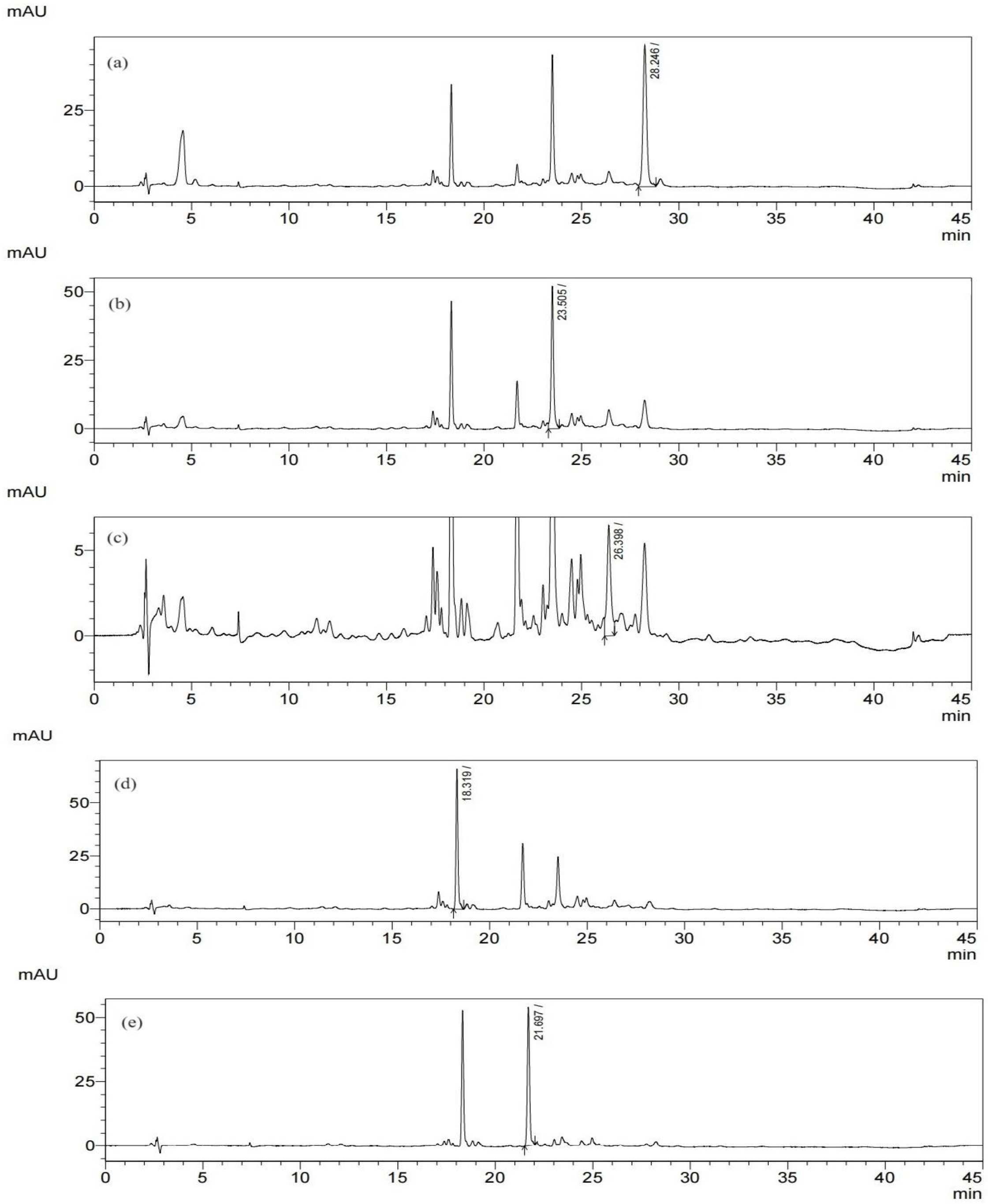

Figure 3.7 High-performance liquid chromatogram (HPLC) chromatogram of carotenoids and chlorophylls of avocado oil at different wavelength, (a) pheophytin *a* at 410 nm, (b)chlorophyll *a* at 430 nm, (c) pheophytin *b* at 430 nm, (d) lutein at 446 nm, (e) chlorophyll *b* at 466 nm

Detection occurred simultaneously at specific wavelengths according to their maximum absorbance: 410 nm for pheophytin *a*, 430 nm for chlorophyll *a*, 435 nm for pheophytin *b*, 446 nm for lutein and other xanthophylls within the sample, and 466 nm for chlorophyll *b* (Figure 3.7). The quantification via HPLC employed the external standard method. Standard curves were generated through linear regression analysis, correlating HPLC peak absorbance area with the concentration of the standard. The obtained results were reported as milligrams per kilogram of avocado oil.

3.7.4 Sterol profile

The determination of sterol profile was based on the gas chromatography-mass spectrometry (GC-MS) method proposed by Tan *et al.* (2018a) and Caligiani *et al.* (2010a), which was mainly focused on four sterols, including campesterol, stigmasterol, β-sitosterol and Δ5-Avenasterol.

3.7.4.1Reagents

- Potassium hydroxide (KOH) ≥ 86.2%: Analytical reagent grade, Fisher Scientific, United Kingdom
- Ethanol absolute: LabServ Analytical, Reagent Grade, New Zealand.
- Pyrogallol: Analytical standard, Sigma-Aldrich, USA
- Cyclohexane≥99.0%: Analytical reagent, Univar, Asia Pacific Specialty Chemicals Ltd, New Zealand
- Pyridine≥99.9%: CHROMASOLVPlus®, HPLC grade, Sigma-Aldrich, USA
- 5α-chloestan-3β-ol≥95.0%: Analytical standard, Sigma-Aldrich, USA
- *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (with1%trimethylchlorosilane (TMCS)): Analytical standard, Sigma-Aldrich, USA

3.7.4.2 Reagent preparation

Derivatization (silylating) reagents: A 1:1 volume ratio mixture of pyridine and BSTFA (with 1% TMCS).

85% w/v Potassium hydroxide (KOH) solution: KOH (8.5 g) was dissolved in MilliQ water in a 10 mL volumetric flask. After it was completely dissolved after using an ultrasonicating water bath, the solution was made up to 10 mL.

Internal standard solution: Twenty milligrams of 5α-chloestan-3β-ol was dissolved into 10 mL ethanol in a volumetric flask.

3.7.4.3 Sample preparation and extraction

The avocado oil sample was precisely weighed to 0.2 g and placed into a preparation glass test tube, to which was added 20 μL of the internal standard solution of 5α-chloestan-3β-ol. Subsequently, 8 mL ethanol/pyrogallol solution was added, followed by the addition of 0.5 mL of 85% KOH solution. The test tube was then capped and swirled for a few seconds. The prepared tube was placed into a water bath at 80 ± 1°C, with intermittent shaking for a duration of 8 minutes. After the extraction, the tube was rapidly cooled using cold tap water. After cooling, 12 mL MilliQ water and 20 mL cyclohexane was immediately added to the tube and the tube mixed well by swirling to ensure homogeneity. The resultant solution was allowed to stand for three minutes until complete phase separation had taken place. The supernatant was collected into a round bottom flask carefully, the solution in the flask was evaporated to dryness in a rotary evaporator (Buchi, Switzerland) at 20°C. The residue obtained was then redissolved in 400 μL of derivatization reagent and placed in a sample vial ready for GC-MS injection.

3.7.1.4 GC-MS operating conditions

The oil samples were analysed by GC-MS 2010 Plus (Shimadzu, Japan) with a Rtx-5 60m x 0.25 mm ID, 0.25 μm thickness column (Restek, Pennsylvania, USA). The column temperature was set at 200°C without holding, then heated to 290°C and held for 5 min. The injection was set to 1 µL at 270°C with 1:10 split, carrier gas was He (1 mL/min) and the run time was 50 minutes in total. MS analysis utilized an ion source temperature of 235°C and an interface temperature of 290°C. Electron energy was set to 70 eV. Acquisition mode was set as scan over the range of 45-550 m/z at a sample rate of 0.2 scans/s.

3.7.1.5 Results analysis

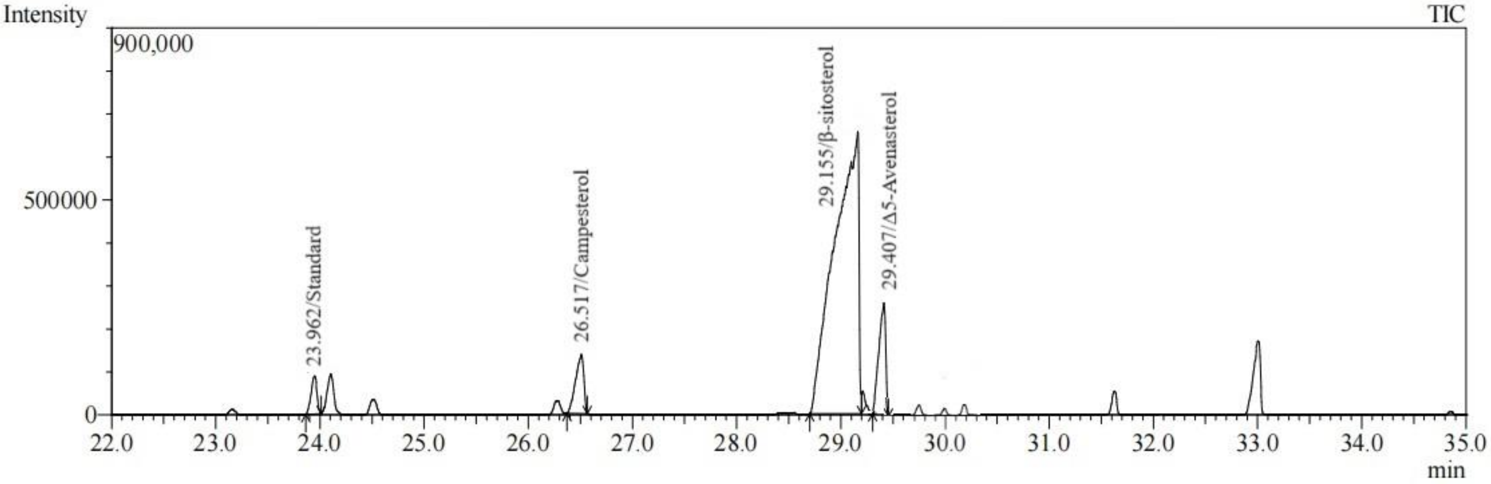

Figure 3.8 A typical GC-MS chromatogram of sterols of avocado oil

A typical GC-MS chromatogram obtained for avocado oil is shown in Figure 3.8. The peak area of different sterols and the internal standard was quantified. The concentration of internal standard was known, therefore the concentration of sterols was calculated using Equation 3.15.

$$C_{sterol}\ (mg/kg) = \frac{C_{standard} \times A_{standard}}{A_{sterol}} \qquad \text{Equation 3.15}$$

Where:

C_{sterol} is the concentration of each sterol (mg kg^{-1} oil);

$C_{standard}$ is the concentration of internal standard (mg kg^{-1} oil);

A_{sterol} is the peak area of each sterol;

$A_{standard}$ is the peak area of internal standard.

3.8 Statistical Analysis

Each experiment and analytical measurement were conducted with at least three replicates to ensure the reliability of the results. Data analysis procedures encompassed the utilization of Microsoft Excel for tasks such as linear regression, computation of mean values and standard error mean. The generation of graphical representations was accomplished using Sigma Plot after the completion of data analysis.

Statistical analysis was conducted using Minitab 17 Statistical Software. To ascertain significant distinctions among samples ($p < 0.05$), a one-way ANOVA followed by a Tukey test was employed. Sample variability was assessed by calculating standard errors of the mean, which was reported as mean ± standard error (SE), with the sample size n.

Chapter 4: Chemical composition of avocado oil with different treatments

In commercial cold-pressed avocado oil extraction, avocado peels and seeds are often discarded as by-products. It has been discovered that skin and seed contain a significant amount of fat-soluble phenolic compounds and pigments. These substances are believed to be beneficial to the human body and may contribute to the storage stability of avocado oil (Cárdenas-Castro *et al.*, 2023; Ferreira & Santos, 2022; Salazar-López *et al.*, 2020; Tesfaye *et al.*, 2022). To investigate the effect of skin and seed with pulp during malaxing on the quality and storage stability of extra virgin avocado oil, from early season and late season avocado fruits three different starting materials were used for oil extraction (Section 3.4.1). These treatments included 'Flesh only' (100% flesh with 10% skin), 'Flesh and skin' (100% flesh + 100% skin), and 'Whole fruit' (100% flesh + 100% skin + 100% seed). After extraction, the avocado oil was immediately nitrogen flushed and frozen at -80°C. This process was to ensure no changes in chemical composition of the oil which were analysed at a later time. The time point that oil was extracted was referred to as time zero, which also served as the first sampling point for the storage trial. This chapter will cover the analysis of the chemical composition of avocado oils extracted from different treatments at time zero to investigate the impact of incorporating avocado skin and seed on the composition of avocado oil.

4.1 Dry matter, oil yield and total oil of avocado fruits

4.1.1 Dry matter of avocado fruits

The maturity of avocado fruit significantly influences its dry matter content, with fruit harvested later in the season having a higher dry matter content (Woolf *et al.*, 2009). The increased dry matter content is strongly correlated with oil yield (Lee *et al.*, 1983; Pak *et al.*, 2003; Woolf *et al.*, 2009; Yang *et al.*, 2020). Therefore, measuring the dry matter before oil extraction can serve as a prediction of subsequent oil yield.

The early season and late season avocados used in the experiment were harvested in August 2022 and July 2022 (Section 3.1.1). Although both harvests of fruit turned black after ripening, there were obvious differences in their appearance while still on the tree (Figure 4.1). The avocados from the early season were an emerald green colour, whereas the late season fruits had started to change colour and were darkening to purple/black, but were still unripe (firm).

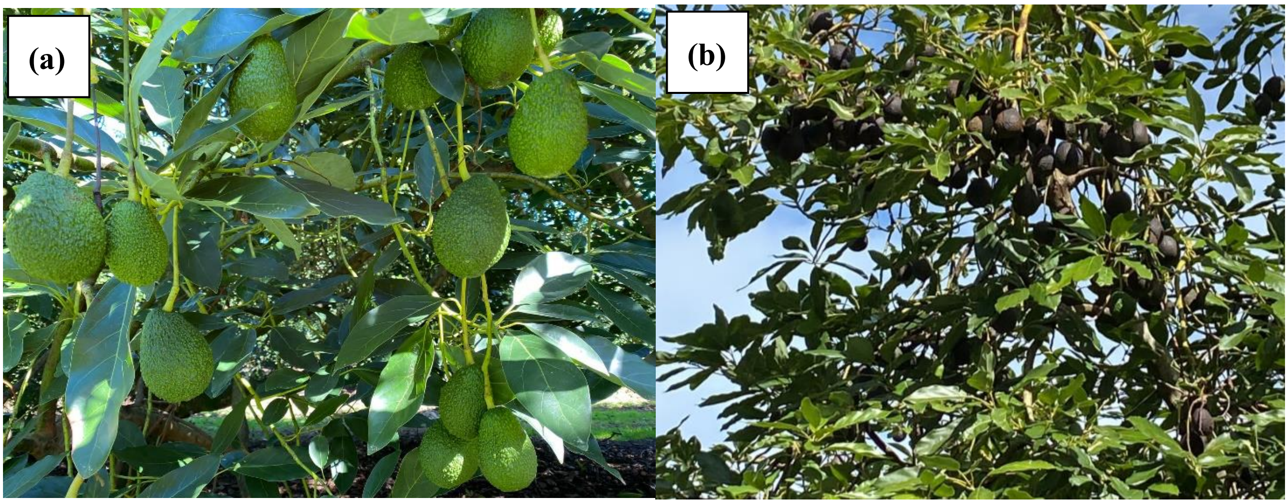

Figure 4.1 Images of (a) early season (August 2022) and (b) late season avocado fruits (July 2022) before harvesting from Waiau Pa, Auckland.

The dry matter content was determined within one day of harvest (Section 3.2.1) and on the different sections of the fruit after ripening (Section 3.3). According to the results in Table 4.1, the dry matter of unripe late season avocados (33.8%) was higher than that of fresh early season avocados (26.5%), which aligned with what is expected for avocados (Woolf *et al.*, 2009; Yang *et al.*, 2020; Yang *et al.*, 2019). Similarly, the dry matter of flesh in ripe fruits did not show significant difference with the unripe fruit and all the tissues of ripe fruits had higher dry matter content for the late season fruits than early season fruits.

Table 4.1. The dry matter content of avocado fruits (mean ± SEM, n=20)

	Dry matter (% g/g wet tissue)			
	Unripe fruit	**Ripe fruit**		
	Whole fruit plug	Flesh	Skin	Seed
Early season (August 2022, NZ)	26.5 ± 0.7	27.3 ± 0.3	28.6 ± 0.3	37.9 ± 0.2
Late season (July 2022, NZ)	33.8 ± 0.5	33.4 ± 0.1	29.0 ± 0.6	43.1 ± 0.1

4.1.2 The total oil content of pulp, skin and seed of avocado fruits

To investigate the total oil content of the pulp, peel, and seed separately, different components of the fruits were ground and dried in an oven (Section 3.3). After Soxhlet extraction the oil content was found to have increased in the flesh and seed components from early to late season. The seed had the lowest oil yield (early 0.52% and late 1.07%), followed by the skin (no significant change between early 8.78% and late 7.56%), and the flesh (early 17.88% and late 23.68%) had the highest oil content (Table 4.2).

Table 4.2. Total oil of avocado pulp, skin and seed after ripening (mean ± SEM, n=6)

	Total oil content % (oil g/g wet tissue)		
	Flesh	Skin	Seed
Early season (August 2022, NZ)	17.88 ± 0.30	8.78 ± 0.17	0.52 ± 0.04
Late season (July 2022, NZ)	23.68 ± 0.01	7.56 ± 0.08	1.07 ± 0.03

4.1.3 Cold-pressed oil yield of avocado fruits

The cold-pressed oil from the three treatments flesh only, flesh and skin and whole fruit is shown in Figure 4.2. It can be observed that the colour of the oil becomes darker in flesh and skin oil and whole fruit oil, indicating that more pigments are likely to have been extracted into the oil during the extraction process. The oil yield of fruits decreased with the addition of components, skin and seed to the flesh in the malaxer (Table 4.3).

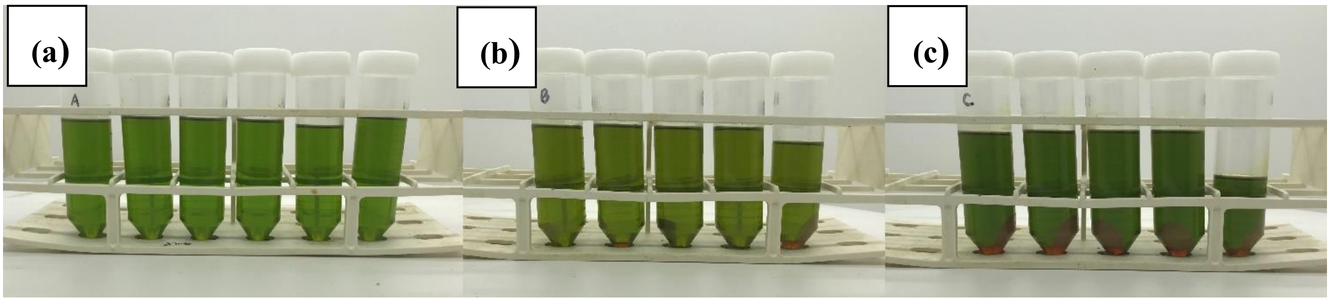

Figure 4.2. Images of the total amount of cold-pressed oil per malaxer extracted from late season avocado fruits (a) Flesh only (b) Flesh and skin (c) Whole fruit.

Regardless if early season or late season fruits were extracted, flesh only extraction resulted in the highest oil yield (early 11.18% and late 16.69%), followed by flesh and skin (early 5.50% and late 14.60%), while whole fruit extracted resulted in the lowest oil yield (early 3.82% and late 10.90%). This indicated that the addition of skin and seed reduced oil extraction yields on a per malaxer basis (Table 4.3).

Table 4.3. Cold-pressed oil yield from malaxers with different tissues of avocado fruit (mean ± SEM, n=8)

	Oil yield % (oil g/g wet tissue)		
	Flesh only	Flesh and skin	Whole fruit
Early season (August 2022, NZ)	11.18 ± 0.87	5.50 ± 1.00	3.82 ± 0.67
Late season (July 2022, NZ)	16.69 ± 0.52	14.60 ± 0.45	10.90 ± 0.45

The oil yields for all three treatments for late season fruits were significantly greater ($p < 0.05$) than those of early season fruits, correlating positively with the amount of dry matter, although the dry matter of skin did not increase. This further validates that the oil yield of avocados increases with the higher fruit maturity, which agreed with previous cold-pressed avocado oil results (Yang *et al.*, 2020).

Moreover, the flesh only treatment resulted in the highest oil yield. As each malaxer contained the same mass of flesh, skin and seed, the replacement of flesh with skin or seed resulted in a lower total oil yield of avocado oil, as there was less total oil available to extract. As shown in Table 4.2 the flesh contains the most oil therefore adding skin and seed to the malaxer reduces the oil yield per malaxer. This will affect the volume of oil collected per malaxer.

4.1.4 Oil quality at time zero after extraction

4.1.4.1 Peroxide value

Peroxide value (PV) is an indicator of the primary oxidation products, which gives a crude measurement of early oil oxidation (Green & Wang, 2022b). As shown in Table 4.4, oil from the same harvest did not show significant difference among different treatments ($p > 0.05$). However, the PV of early season oils were significantly higher than that of late season oils with the same treatment ($p < 0.05$). But the difference between early and late season PV values was on average 1 meq/kg oil, and the early season oils were still lower than the recommended maximum of PV for extra virgin oil reported by Woolf *et al.* (2009) which is 4.0 meq/kg oil. Adding skin and seed did not increase PV in oils.

Table 4.4. PV of avocado oil at time zero (mean ± SEM, n=3)

	Early season fruit oil (meq peroxide/kg oil)	**Late season fruit oil** (meq peroxide/kg oil)
Flesh only	2.8 ± 0.5	1.6 ± 0.2
Flesh and skin	2.7 ± 0.2	1.7 ± 0.8
Whole fruit	2.6 ± 0.1	1.7 ± 0.1

4.1.4.2 %Free Fatty Acids

Free fatty acids (FFA) serves as a crucial indicator for assessing the quality of oil and its limits are often incorporated into grade standards (Codex Alimentarius Commission, 2021; International Olive Council, 2019b). A high FFA value suggests fermentation or potential damage of the fruits, leading to lower-quality oil due to increased separation of fatty acids from the triacylglycerol molecule (TAG) through the process of hydrolysis (Woolf *et al.*, 2009).

Table 4.5. FFA of avocado oil at time zero (mean ± SEM, n=3)

	Early season fruit oil (% as oleic acid)	**Late season fruit oil** (% as oleic acid)
Flesh only	0.30 ± 0.01	0.29 ± 0.02
Flesh and skin	0.23 ± 0.02	0.26 ± 0.03
Whole fruit	0.24 ± 0.01	0.26 ± 0.03

The FFA values at time zero of different avocado oils are shown in Table 4.5. The initial FFA values among all the oils did not show significant differences ($p > 0.05$), ranging from 0.23% to 0.30% (as oleic acid). These results met the extra virgin avocado oil standard proposed by Woolf *et al.* (2009), less than 0.5% as oleic acid. It meant fatty acids did not undergo significant hydrolysis during malaxing, meanwhile adding skin and seed did not have a significant effect on FFA ($p > 0.05$), which agreed with previous research (Wong *et al.*, 2011).

4.1.4.3 Specific Extinction Coefficients K_{232} and K_{270}

Utilizing UV absorbance is an alternative and common method to monitor oil oxidation (Barriuso *et al.*, 2013; Zhang *et al.*, 2023). The absorption at wavelength of 232 nm (referred to as K_{232}) serves as an indicator for peroxides, representing primary oxidation products, specifically conjugated dienes. Secondary oxidation products are often generated under higher temperature conditions, which are measured at a wavelength of 270 nm (referred to as K_{270}) (Angerosa *et al.*, 2006; Kiritsakis *et al.*, 2002). The delta K value is a dimension-less parameter computed by comparing other UV absorbances. It was introduced to identify the presence of refined oil with a threshold set at values below 0.01 (Vossen, 2007). Delta K values are not reported as all the oils are cold-pressed extracted at 50 ± 1°C and they were not refined.

As shown in Table 4.6, it was found at time zero that the K_{232} value of early season avocado oil was highest for whole fruit oil (2.78 ± 0.04), followed by flesh and skin oil (2.07 ± 0.01), and the lowest for flesh only oil (1.91 ± 0.03). Similarly in the late season, whole fruit oil (3.74 ± 0.01) exhibited the highest K_{232} value, followed by flesh and skin oil (3.20 ± 0.01), and then flesh only oil (2.38 ± 0.01). As for K_{270}, although flesh only oil did not show significant difference with flesh and skin oil ($p > 0.05$) for both seasons, the whole fruit oil was significantly higher than the oil from the two other treatments ($p < 0.05$). As K_{232} and K_{270} have not been widely used in avocado oil oxidation evaluation, thus it is not part of proposed CODEX standard so far. According to the extra virgin olive oil standard, K_{232} and K_{270} should be lower than 2.5 and 0.22, respectively (International Olive Council, 2019b). Compared to PV, the differences in K values indicated that the primary oxidation products are more significant in the oils which had been extracted with skin or both skin and seed. The secondary oxidation products were below the recommended limits base on K_{270} values.

Table 4.6. K values of avocado oil at time zero (mean ± SEM, n=3)

	K_{232}	K_{270}
Early season - Flesh only	1.91 ± 0.03	0.06 ± 0.01
Early season - Flesh and skin	2.07 ± 0.01	0.06 ± 0.01
Early season - Whole fruit	2.78 ± 0.04	0.20 ± 0.01
Late season - Flesh only	2.38 ± 0.01	0.07 ± 0.01
Late season - Flesh and skin	3.20 ± 0.01	0.08 ± 0.01
Late season - Whole fruit	3.74 ± 0.01	0.15 ± 0.01

4.2 Fatty acid composition of avocado oil extracted with different treatments

The fatty acid profile of avocado oil was determined by gas chromatography (GC) method (Section 3.7.1). The GC chromatograms shown in Figure 4.3 show the fatty acid profile for early season and late season avocado oil at time zero (no storage). According to the retention times and based on the previous references (Milinsk *et al.*, 2008; Reddy *et al.*, 2012; Tan, 2019), it was found that the main fatty acids in avocado oil were palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid. Cis-vaccenic acid, recently reported by Green and Wang (2022a), was also identified.

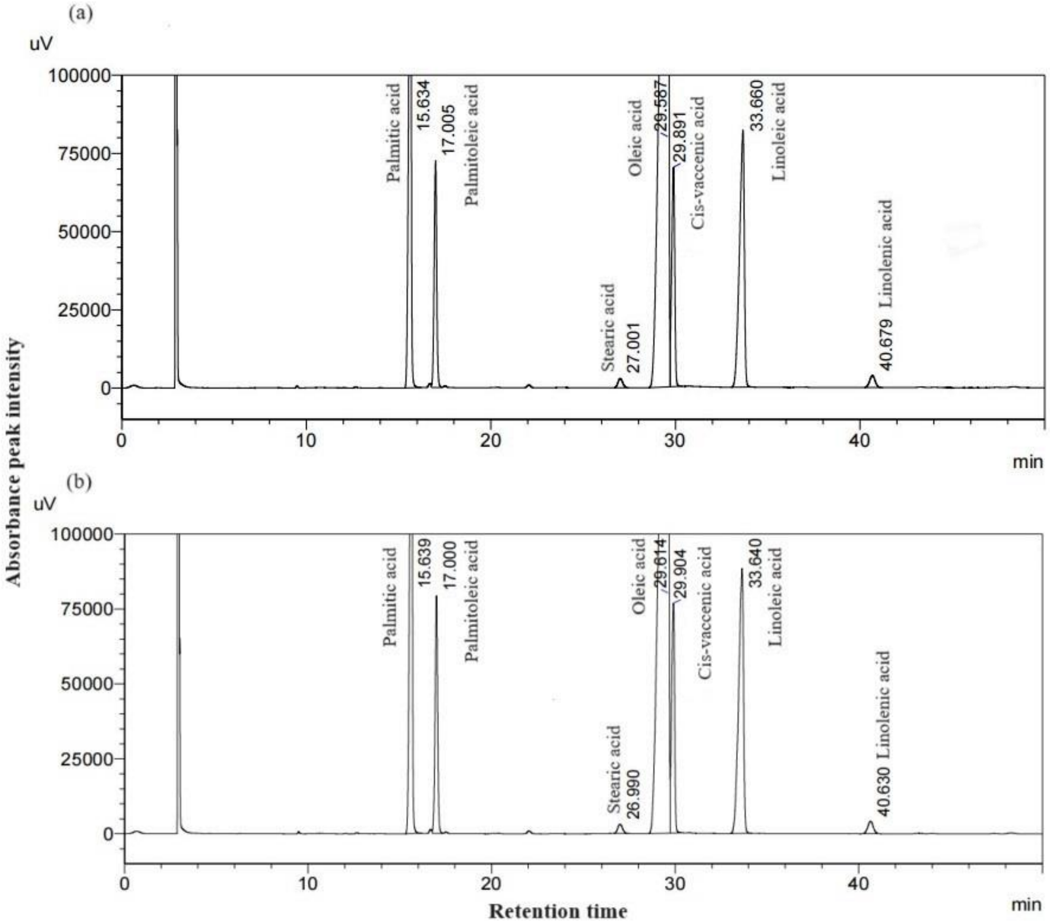

Figure 4.3. Representative GC chromatograms of (a) early season and (b) late season avocado oil at time zero

The percentage of different fatty acids were calculated based on their peak areas as shown in Figure 4.4. The fatty acid profile for flesh only oil for both seasons were very similar. The palmitic acid, palmitoleic acid, stearic acid, oleic acid, cis-vaccenic acid, linoleic acid and linolenic acid were found to be 14.4 ± 0.01%, 5.4 ± 0.01%, 0.4 ± 0.01%, 61.3 ± 0.01%, 6.3 ± 0.02%, 11.6 ± 0.01% and 0.6 ± 0.01% %, respectively.

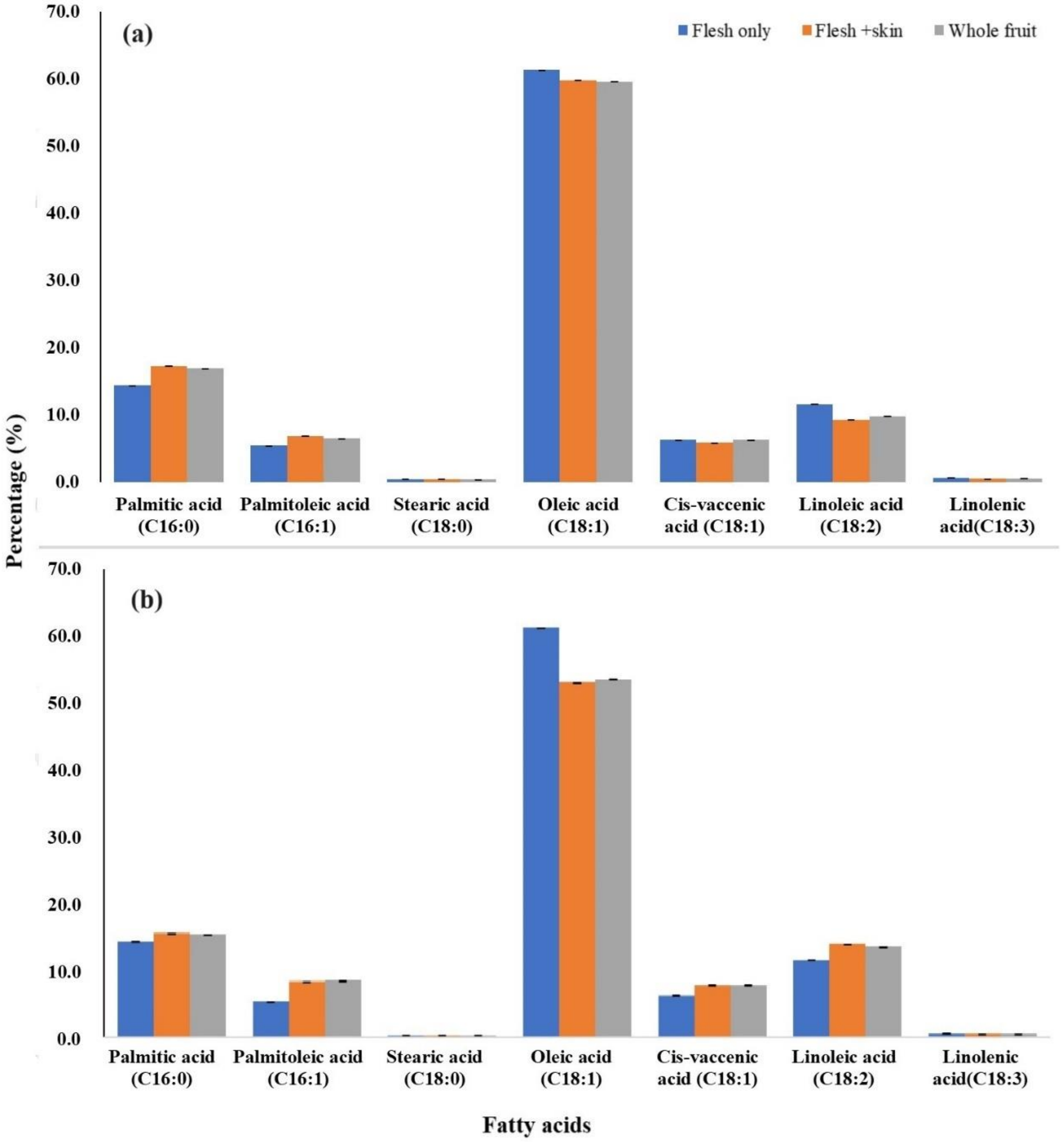

Figure 4.4. Fatty acid profile of (a) early season and (b) late season avocado oil under three treatments at time zero. Percentage of each fatty acid present. (mean ± SEM n=3)

As for flesh and skin and whole fruit extracted oils, they had a higher proportion of palmitic acid, stearic acid, and oleic acid but a lower proportion of palmitoleic acid, cis-vaccenic acid, linoleic acid, and linolenic acid in early season fruit oil compared to the late season oil, the differences are significant ($p < 0.05$). Fatty acid compositions are also reported in the Appendix I.

The results from the fatty acid profiles showed that addition of peel and seed during malaxing did not have a significant impact ($p > 0.05$) on the fatty acid profile in oil. The proportions of the main fatty acids remained relatively unchanged. Compared to the current CODEX standards, all fatty acid values in this study conformed to the proposed criteria (Codex Alimentarius Commission, 2021).

4.3 Phenolics compounds of avocado oil extracted with different treatments

4.3.1 Total phenolics

Avocado peel and seed contain a significant amount of phenolic compounds (Dabas *et al.*, 2013; Kosińska *et al.*, 2012; Morais *et al.*, 2015; Santana *et al.*, 2019). Regular consumption of foods rich in these types of phenolic compounds has been associated with health benefits because they possess antioxidant, anti-proliferative, anti-inflammatory, and anti-mutagenic properties (Alkhalaf *et al.*, 2019; Tavsan & Kayali, 2019). Avocado oil extracted with skin and seed could potentially contain high levels of phenolic compounds. The Folin-Ciocalteu method is the most commonly used method for determining the total phenolic content (Section 3.6.4).

At time zero (Table 4.7), the total phenolic content of oil from different treatments from highest to lowest was observed in whole fruit oil (early 31.7 and late 25.9 mg kg^{-1}), then in flesh and skin oil (early 20.7 and late 18.2 mg kg^{-1}), and then flesh only oil (early 12.0 and late 7.9 mg kg^{-1}). The early season oil consistently contained a significantly higher concentration of total phenolics compared to the late season oil ($p < 0.05$), even in the flesh only oil. These results indicate that a portion of the phenolic compounds from the peel and seed was extracted into the oil during the cold-pressed extraction process. The total phenolic content extracted in avocado oil increased after malaxing with the extra skin and seed.

Table 4.7. Total phenolic content of avocado oil at time zero (mean ± SEM, n=3)

	Early season fruit oil (mg kg^{-1} oil, caffeic acid equivalent)	**Late season fruit oil** (mg kg^{-1} oil, caffeic acid equivalent)
Flesh only	12.03 ± 0.63	7.92 ± 0.15
Flesh and skin	20.70 ± 0.47	18.25 ± 0.11
Whole fruit	31.70 ± 0.86	25.94 ± 0.17

4.3.2 Individual phenolics

The exploration of individual phenolics was conducted with reverse phase high-performance liquid chromatography (HPLC) with detection at 280 nm. Individual phenolics were identified by comparing to external phenolic standards (Section 3.7.2). Representative HPLC chromatograms are shown in Figure 4.5. Each peak represents a specific phenolic compound, and the peak areas were used to quantify any known phenolic compounds. With the addition of peel and seed, a greater variety of phenolic compounds were observed in the oils extracted and the quantity of phenolics had increased as indicated by the increase in peak areas. This further supports the findings that the addition of other fruit components increases the content of phenolic compounds in the oil, consistent with the total phenolic content findings (Section 4.3.1).

By using external standards and standard curves, the concentration of each known phenolic standard compound was obtained as shown in Table 4.8. Since a number of phenolic peaks could not be identified and quantified with the known external standards, these peaks were quantified based on the caffeic standard curve and reported as concentration of caffeic acid equivalent. The results show that under the same extraction treatment, late season oil contained a greater variety of phenolic compounds compared to early season oil. The total phenolic content for each treatment was higher in early-season oils. This indicated that as the fruit matured, the phenolic profile in the different components in the fruit increase changed while their overall content decreased (Table 4.7). This is consistent with the findings for total phenolic content (Section 4.3.1).

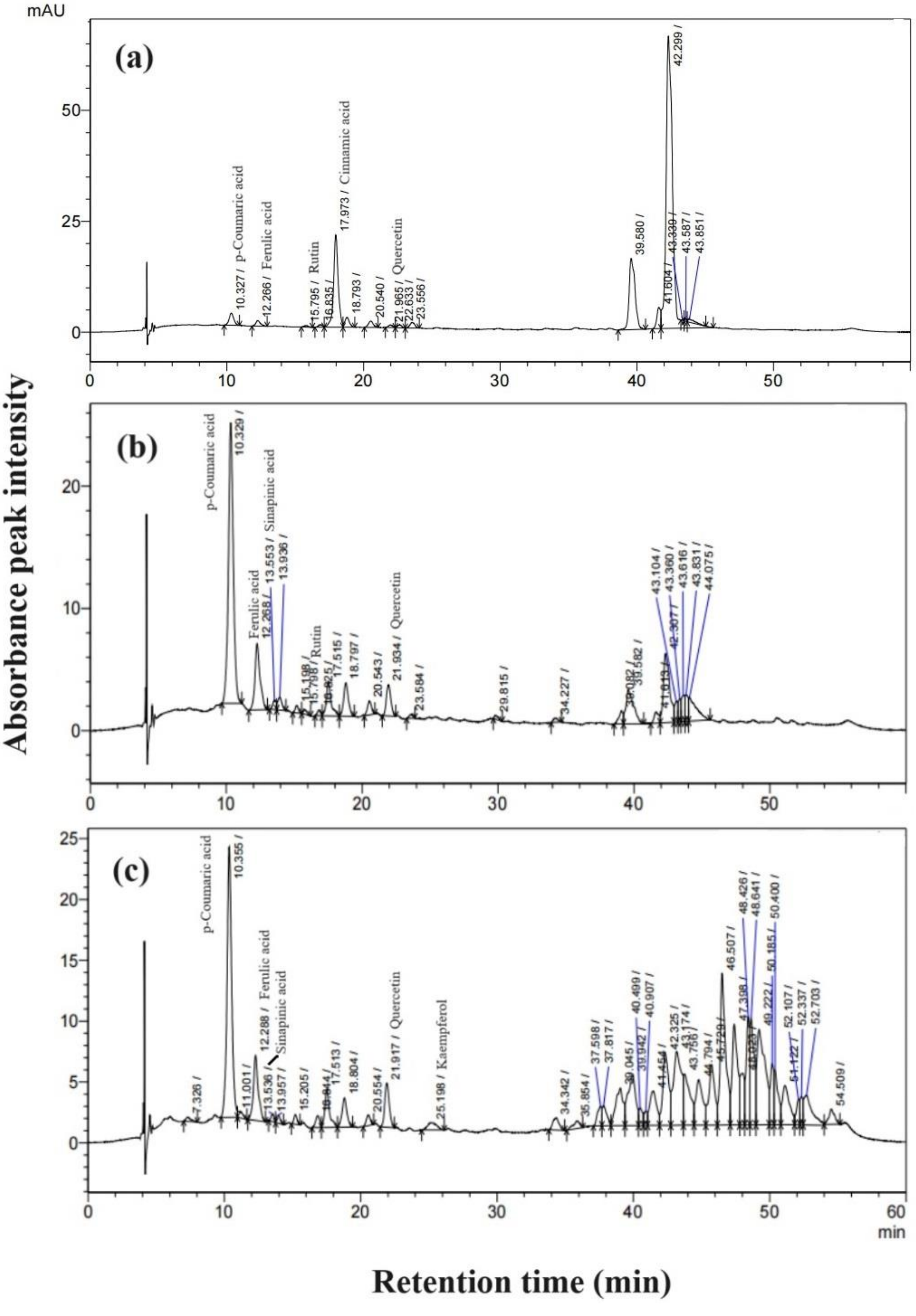

Figure 4.5. Representative HPLC chromatograms of individual phenolics in (a)Flesh only, (b) Flesh and skin and (c) Whole fruit avocado oil

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Table 4.8. Individual phenolic compounds concentration (mg kg^{-1} oil) in avocado oil based on known external standards (known phenolics) and expressed as caffeic acid equivalent (unknown phenolics) (mean ± SEM, n=3).

Retention time (mins)	Phenolic compounds	Flesh only		Flesh +skin		Whole fruit	
		Early	Late	Early	Late	Early	Late
5.64	Gentistic acid	ND	ND	ND	ND	ND	1.40±0.01
6.85	Vanillic acid	ND	ND	ND	0.12±0.03	ND	0.09±0.03
7.33	Unknown 1	ND	ND	ND	ND	0.08	ND
7.73	Caffeic acid	ND	ND	ND	0.06±0.01	ND	0.06±0.01
8.88	Epicatechin	ND	ND	ND	0.14±0.04	ND	0.13±0.03
10.33	p-Coumaric acid	0.46±0.01	0.11±0.01	4.22±0.16	0.25±0.01	3.83±0.16	0.85±0.03
11.00	Unknown 2	ND	ND	ND	ND	0.12±0.01	ND
11.24	Benzoic acid	ND	0.35±0.07	ND	1.14±0.05	ND	2.44±0.60
12.25	Ferulic acid	0.34±0.01	0.26±0.01	1.75±0.04	0.40±0.13	1.59±0.03	0.90±0.02
12.29	Unknown 3	ND	0.08±0.01	ND	ND	ND	ND
13.49	Sinapinic acid	ND	0.17±0.01	0.44±0.08	1.07±0.07	0.30±0.01	0.81±0.04
13.92	Unknown 4	ND	ND	0.21±0.01	0.11±0.01	0.11±0.01	0.12±0.01
14.75	Unknown 5	ND	ND	ND	0.09±0.01	ND	0.15±0.01
15.16	Unknown 6	ND	ND	0.09±0.01	0.15±0.01	0.11±0.01	0.17±0.01
15.75	Rutin	0.59±0.01	0.77±0.03	0.52±0.02	0.97±0.01	ND	0.93±0.02
16.83	Unknown 7	0.14±0.03	0.25±0.01	0.08±0.01	0.10±0.01	0.19±0.01	0.20±0.01
17.49	Unknown 8	ND	0.11±0.01	0.65±0.01	0.16±0.02	0.69±0.01	0.44±0.01
17.94	Cinnamic acid	2.29±0.05	0.85±0.01	ND	0.02±0.01	ND	0.09±0.02
18.79	Unknown 9	0.47±0.01	0.20±0.01	0.62±0.01	0.19±0.01	0.54±0.01	0.58±0.01
20.03	Unknown 10	ND	0.09±0.01	ND	0.18±0.02	ND	0.12±0.01
20.36	Unknown 11	ND	0.08±0.01	ND	0.19±0.01	ND	0.24±0.01
20.55	Unknown 12	0.33±0.01	ND	0.22±0.01	ND	0.16±0.01	ND
21.12	Unknown 13	ND	ND	ND	0.11±0.01	ND	0.22±0.01
21.88	Quercetin	0.56±0.01	0.25±0.06	2.75±0.05	1.30±0.28	3.81±0.01	2.29±0.18
22.60	Unknown 14	0.14±0.01	0.03±0.01	ND	0.02±0.01	ND	0.02±0.01
23.55	Unknown 15	0.30±0.01	0.17±0.01	0.06±0.01	0.14±0.01	ND	0.13±0.04
24.26	Unknown 16	ND	0.22±0.01	ND	0.12±0.01	ND	0.05±0.01
24.39	Unknown 17	ND	ND	ND	ND	ND	0.11±0.01
24.89	Unknown 18	ND	ND	ND	ND	ND	0.05±0.01
25.07	Kaempferol	ND	ND	ND	0.15±0.01	0.61±0.08	0.36±0.07
25.99	Unknown 19	ND	0.08±0.01	ND	0.09±0.01	ND	0.10±0.02
26.35	Unknown 20	ND	ND	ND	0.02±0.01	ND	0.04±0.01
29.83	Unknown 21	ND	ND	ND	0.04±0.01	ND	0.05±0.01
34.34	Unknown 22	ND	ND	0.08±0.01	ND	0.29±0.01	ND
35.90	Unknown 23	ND	ND	ND	ND	0.19±0.01	0.04±0.01
36.48	Unknown 24	ND	ND	ND	0.05±0.01	ND	0.12±0.01
36.83	Unknown 25	ND	ND	ND	ND	ND	0.04±0.01
37.40	Unknown 26	ND	ND	ND	0.03±0.01	ND	0.03±0.01
37.57	Unknown 27	ND	ND	ND	ND	0.32±0.01	0.04±0.01

37.87	Unknown 28	ND	ND	ND	ND	0.36±0.01	0.11±0.01
38.85	Unknown 29	ND	ND	ND	ND	ND	0.07±0.01
39.10	Unknown 30	ND	ND	0.21±0.04	0.03±0.01	1.07±0.01	0.11±0.01
39.59	Unknown 31	4.27±0.01	1.60±0.01	1.00±0.01	0.28±0.01	ND	0.31±0.01
39.94	Unknown 32	ND	ND	ND	ND	1.52±0.03	0.18±0.01
40.50	Unknown 33	ND	ND	ND	ND	0.25±0.01	ND
40.91	Unknown 34	ND	ND	ND	ND	0.18±0.01	ND
41.55	Unknown 35	ND	ND	0.21±0.01	ND	0.91±0.04	0.24±0.01
41.67	Unknown 36	0.95±0.01	0.04±0.01	ND	ND	ND	ND
42.33	Unknown 37	18.42±0.06	7.10±0.01	1.64±0.01	1.03±0.01	1.63±0.01	1.35±0.03
43.22	Unknown 38	ND	ND	0.26±0.02	0.17±0.01	2.30±0.02	0.58±0.01
43.36	Unknown 39	ND	ND	0.22±0.01	ND	ND	ND
43.58	Unknown 40	ND	ND	0.33±0.01	0.84±0.01	ND	0.36±0.01
43.66	Unknown 41	1.21±0.09	0.46±0.01	ND	ND	ND	ND
43.83	Unknown 42	ND	0.24±0.01	0.31±0.02	ND	1.28±0.01	0.55±0.01
44.08	Unknown 43	ND	0.62±0.01	0.72±0.01	0.71±0.05	ND	ND
44.85	Unknown 44	ND	ND	ND	ND	1.21±0.01	0.49±0.01
45.77	Unknown 45	ND	ND	ND	ND	1.37±0.02	0.28±0.01
46.54	Unknown 46	ND	ND	ND	ND	3.15±0.06	0.78±0.01
47.45	Unknown 47	ND	ND	ND	ND	2.09±0.04	0.70±0.01
48.11	Unknown 48	ND	ND	ND	ND	0.83±0.01	0.21±0.01
48.47	Unknown 49	ND	ND	ND	ND	1.38±0.01	0.50±0.01
48.70	Unknown 50	ND	ND	ND	ND	1.65±0.01	0.43±0.01
49.31	Unknown 51	ND	ND	ND	ND	2.99±0.08	0.55±0.01
49.60	Unknown 52	ND	ND	ND	ND	ND	0.42±0.01
50.23	Unknown 53	ND	ND	ND	ND	0.81±0.01	0.19±0.01
50.47	Unknown 54	ND	ND	ND	ND	0.80±0.01	0.18±0.01
51.11	Unknown 55	ND	ND	ND	0.03±0.01	1.07±0.01	0.40±0.01
51.61	Unknown 56	ND	0.12±0.01	ND	0.22±0.01	ND	ND
52.12	Unknown 57	ND	ND	ND	ND	0.32±0.01	0.02±0.01
52.34	Unknown 58	ND	ND	ND	ND	0.28±0.01	ND
52.74	Unknown 59	ND	ND	ND	0.04±0.01	0.96±0.01	0.04±0.01
54.60	Unknown 60	ND	ND	ND	ND	0.33±0.01	0.05±0.01
55.70	Unknown 61	ND	0.15±0.03	ND	0.37±0.01	ND	ND
Total phenolics[a]		30.47±0.08	14.41±0.01	16.59±0.03	11.01±0.09	41.68±0.42	22.48±0.53

ND – Not Detected

[a] Sum of each column

With the addition of skin and seed, there was an increase in the concentration of some individual phenolic compounds, for example in early season oil, p-coumaric acid increased from 0.46 ± 0.01 mg kg^{-1} to 4.22 ± 0.16 mg kg^{-1} between flesh only and flesh and skin, respectively (Table 4.8). Meanwhile, in late season oils, benzoic acid increased from 0.35 ± 0.07 mg kg^{-1} to 2.44 ± 0.60 mg kg^{-1} between flesh only and whole fruit, respectively. It is also worth noting that there are instances where the concentration of certain phenolics, such as cinnamic acid, significantly decreased ($p < 0.05$) from 0.85 ± 0.01 mg kg^{-1} to 0.09 ± 0.01 mg kg^{-1} between oils from flesh only and whole fruit in late season oil. The following phenolics compounds were also found in the avocado oils extracted: ferulic acid, sinapinic acid, rutin, and quercetin. After adding skin and seed, gentisic acid, vanillic acid, caffeic acid, epicatechin, and kaempferol were also identified in the oils.

Comparing with the phenolic compounds found in the avocado flesh , skin and seed as reported in previous literature (Jimenez *et al.*, 2021; Kosińska *et al.*, 2012; Melgar *et al.*, 2018; Velderrain-Rodríguez *et al.*, 2021), it was found that phenolic compounds originally only found in the skin and seed were found to be soluble in the avocado oil during the extraction process, including vanillic acid, rutin and kaempferol. The results obtained were similar to the types and concentrations of phenolic compound in avocado oil identified in previous research (Santana *et al.*, 2019).

4.4 Carotenoids (Lutein and β-carotene)

The qualitative and quantitative analysis of the carotenoid lutein was achieved by detection at 446 nm by HPLC (Figure 4.6) using diode array detection (Section 3.7.3). The carotenoid β-carotene was determined by a spectrophotometric method using its specific extinction coefficient in hexane (Section 3.7.3.3).

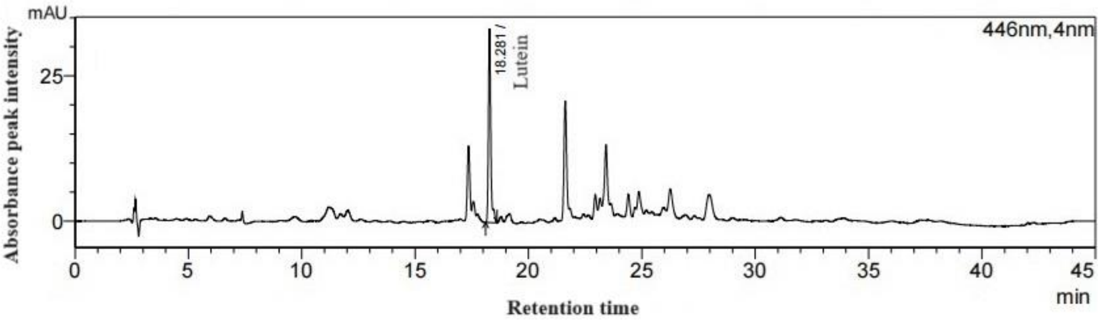


Figure 4.6. HPLC chromatograms of lutein in avocado oil at time zero.

Lutein and β-carotene are the two predominant lipid-soluble carotenoids in avocado oil. From Table 4.9, no significant difference was found in the lutein content between the flesh only oil and flesh and skin oil for both early season and late season ($p > 0.05$), whereas the lutein content in the whole fruit oil was significantly higher than the two other oils for both seasons ($p < 0.05$). Meanwhile, the lutein content did not show significant difference between early season and late season for flesh only and flesh and skin oil ($p > 0.05$), however the lutein content in the whole fruit oil of early season was significantly higher than the whole fruit oil of late season ($p < 0.05$).

Table 4.9. Lutein and β-carotene content of avocado oil at time zero (mean ± SEM, n=3).

	Lutein (mg kg^{-1} oil)	**β-Carotene** (mg kg^{-1} oil)
Early season - Flesh only	3.84 ± 0.01^{b}	7.08 ± 0.03^{b}
Early season - Flesh and skin	3.83 ± 0.01^{b}	9.48 ± 0.03^{c}
Early season - Whole fruit	8.87 ± 0.07^{d}	16.01 ± 0.03^{e}
Late season - Flesh only	3.93 ± 0.02^{b}	2.73 ± 0.09^{a}
Late season - Flesh and skin	3.34 ± 0.10^{a}	13.88 ± 0.09^{d}
Late season - Whole fruit	4.95 ± 0.01^{c}	17.16 ± 0.01^{f}

Values significantly different in the column are noted with different lower case letters $^{a\text{-}f}$.

The β-carotene content in avocado oil significantly increases with the addition of skin and seed ($p < 0.05$) for both seasons, plus there was a higher concentration of β-carotene in oil from late season for flesh and skin and whole fruit oil. This indicated that when seed was included more carotenoids, lutein and β-carotene were extracted from the seed due to its high concentration of carotenoids (Ferreira da Vinha *et al.*, 2020). Early season avocado oil had a higher amount of lutein compared to late season, while late-season avocado oil exhibited a higher concentration of β-Carotene.

4.5 Total chlorophylls

The total chlorophyll content of different oils at time zero was determined using a spectrophotometric method (Section 3.6.6). It was found that the addition of skin resulted in a slight increase in the total chlorophyll content compared to oil from flesh only, but there was a significant increase ($p < 0.05$) in total chlorophyll when oil was extracted from whole fruit (Table 4.10). Compared to previous research, the total chlorophyll content of flesh and skin oil were lower than previously reported by Wong *et al.* (2011) who reported concentrations by 27.5 mg kg^{-1} oil.

Table 4.10. Total chlorophyll content of avocado oil extracted with different treatments at time zero (mean ± SEM, n=3).

	Early season fruit oil (mg kg^{-1} oil)	Late season fruit oil (mg kg^{-1} oil)
Flesh only	14.0 ± 0.1^{a}	13.3 ± 0.1^{a}
Flesh and skin	15.7 ± 0.1^{a}	14.7 ± 0.1^{a}
Whole fruit	36.3 ± 0.2^{c}	21.2 ± 0.1^{b}

Values significantly different in the column are noted with different letters $^{a, b, c}$.

Comparing the avocado oils from early vs late season oils, the flesh only and flesh and skin oil in the early season had slightly higher total chlorophyll content than those in the late season, but this was not significant ($p > 0.05$). For oil extracted from whole fruit contained significantly higher concentrations of total chlorophyll in early season oil compared to the late season ($p < 0.05$). This indicated that oil produced from avocados from the early season contained a higher chlorophyll concentration, plus the addition of skin during the malaxing process did not significantly increase the total chlorophyll content of the oil. With the inclusion of seed, the total chlorophyll increased, but it is unclear where the chlorophyll was from as the seeds are not known to contain high concentrations of chlorophyll.

4.6 Individual chlorophylls by HPLC

The qualitative and quantitative analysis of individual chlorophylls carried out with HPLC and diode array detection using a number of wavelengths (Section 3.7.3). Chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and pheophytin *b*, were identified and quantified using the diode array at the following wavelengths 430 nm, 466 nm, 410nm and 435 nm, respectively (Figure 4.7).

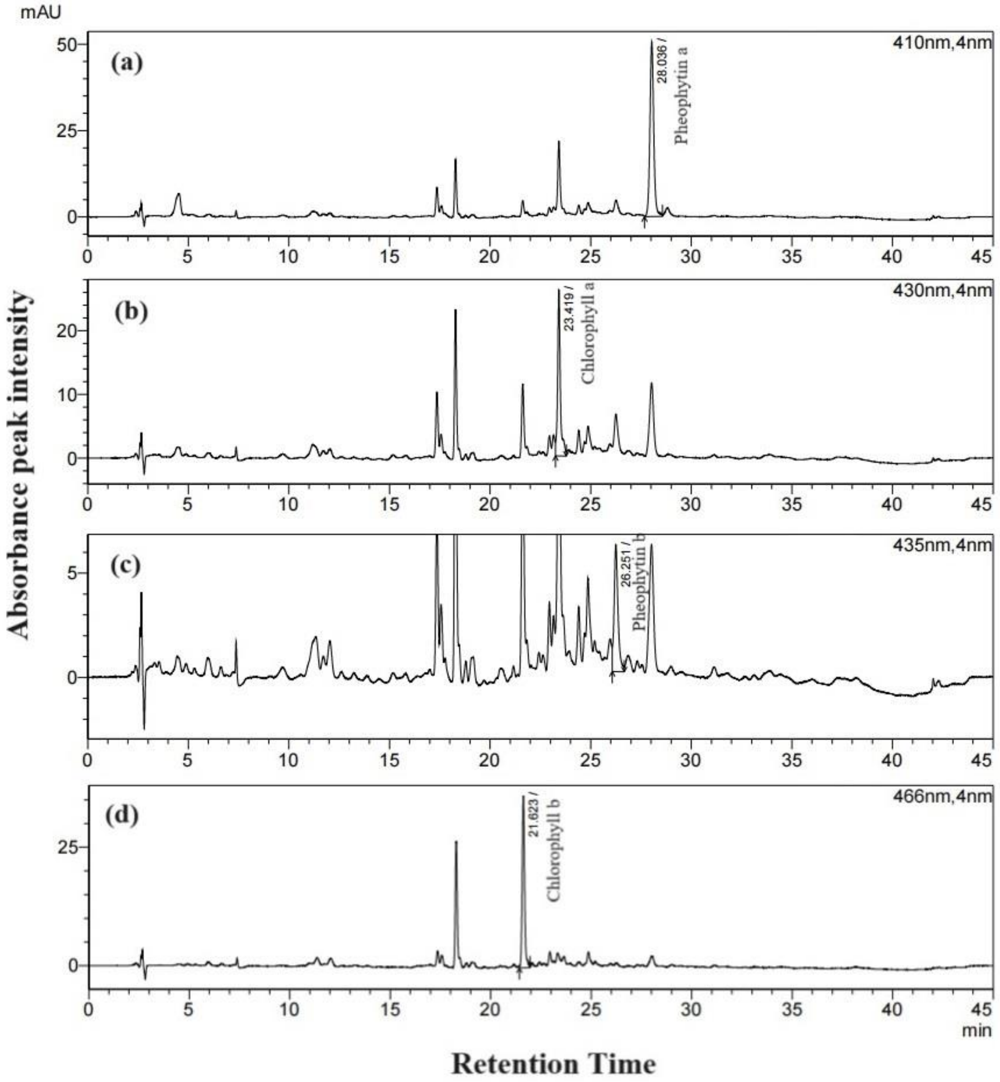


Figure 4.7. HPLC chromatograms of individual chlorophyll in avocado oil at time zero (a) Pheophytin *a*, (b) Chlorophyll *a*, (c) Pheophytin *b*, (d) Chlorophyll *b*

Table 4.11 presents the results for the different chlorophyll pigment concentrations for different avocado oil samples at time zero. It can be found that the flesh only oil from late season exhibited no significant change in chlorophyll *a* and chlorophyll *b* compared with early season flesh only oil ($p > 0.05$), however, there was a significant decrease in the concentration of pheophytin *a* and pheophytin *b* ($p < 0.05$). As for the flesh and skin oil, all four pigment concentrations from late season were significantly higher than that from early season. Although the late season whole fruit oil showed a significant decrease in the content of chlorophyll *a* and chlorophyll *b* compared to early season ($p < 0.05$), the content of pheophytin *a* and pheophytin *b* from whole fruit showed a slight increase.

Table 4.11. Chlorophyll a, chlorophyll b, pheophytin a and pheophytin b content of avocado oil at time zero (mean ± SEM, n=3).

	Chlorophyll *a*	**Chlorophyll *b***	**Pheophytin *a***	**Pheophytin *b***
	(mg kg^{-1} oil)	(mg kg^{-1} oil)	(mg kg^{-1} oil, as chlorophyll *a* equivalent)	(mg kg^{-1} oil, as chlorophyll *b* equivalent)
Early season - Flesh only	17.27 ± 0.01^{c}	9.08 ± 0.01^{b}	7.06 ± 0.04^{b}	0.71 ± 0.09^{ab}
Early season - Flesh + skin	9.47 ± 0.01^{a}	6.34 ± 0.12^{a}	14.79 ± 0.05^{c}	1.26 ± 0.12^{b}
Early season - Whole fruit	20.68 ± 0.62^{d}	19.53 ± 0.23^{e}	27.89 ± 0.10^{e}	3.72 ± 0.05^{d}
Late season - Flesh only	18.17 ± 0.02^{c}	8.89 ± 0.01^{b}	3.86 ± 0.01^{a}	0.39 ± 0.01^{a}
Late season - Flesh + skin	11.40 ± 0.07^{b}	10.38 ± 0.25^{c}	20.48 ± 0.09^{d}	2.07 ± 0.02^{c}
Late season - Whole fruit	12.30 ± 0.01^{b}	15.00 ± 0.54^{d}	32.65 ± 0.14^{f}	3.88 ± 0.02^{d}

Values significantly different in the column are noted with different letters $^{a\text{-}f}$.

Comparing oils only from the same season but across treatment types, it can be found that in flesh and skin oil, despite a significant decrease in the content of chlorophyll *a* and chlorophyll *b* compared to flesh only oil, there was a notable increase in the levels of pheophytin *a* and pheophytin *b* ($p < 0.05$). Additionally, in whole fruit oil, all four pigments showed a significant increase compared to flesh only oil ($p < 0.05$).

The total chlorophyll concentration (mg kg^{-1} oil) reported in Section 4.5 was found to be less than the sum of the individual chlorophyll pigments reported in Table 4.12. This difference could be attributed to the fact that total chlorophyll measurement involves only one wavelength, whereas individual chlorophylls were measured at their respective maximum absorption wavelengths. The conclusion that the addition of fruit skin increased the pheophytin *a* and pheophytin *b* content but decreased the concentration of chlorophyll *a* and chlorophyll *b* in avocado oil did not agree with Ashton *et al.* (2006)'s research findings. But extracting oil from the whole fruit enhanced the content of chlorophylls presented in avocado oil.

The addition of skin though slightly increased the total chlorophyll concentration, this was not a significant increase in chlorophylls in avocado oil. This could be attributed to the fact that the skin of the late season fruit had already started to change colour when they were harvested. Cox *et al.* (2004) reported the increase in anthocyanins and decrease in chlorophylls in the skin with colour changes during ripening. Therefore, the skin did not have carotenoids or chlorophylls present but likely to contain more anthocyanins and less chlorophylls.

4.7 Sterol profile

The phytosterol determination in avocado oils was achieved using GC-MS (Section 3.7.4). Only the major sterols in avocado oil, including campesterol, stigmasterol, β-sitosterol, and Δ5-Avenasterol were identified. The sterol results obtained are shown in Table 4.12, as % of each sterol present and the actual concentrations determined are shown in Table 4.13.

Table 4.12. Sterol profile (%) of avocado oils at time zero (mean ± SEM, n=3).

	Flesh only		Flesh and skin		Whole fruit	
	early	late	early	late	early	late
Campesterol	6.6 ± 0.1[b]	4.3 ± 0.1[a]	6.4 ± 0.1[b]	4.5 ± 0.1[a]	6.2 ± 0.1[b]	4.5 ± 0.1[a]
Stigmasterol	ND	ND	ND	ND	ND	ND
β-sitosterol	84.8 ± 0.1[f]	88.0 ± 0.1[g]	85.3 ± 0.1[f]	88.5 ± 0.1[g]	84.3 ± 0.1[f]	88.3 ± 0.1[g]
Δ5-Avenasterol	8.6 ± 0.1[d]	7.6 ± 0.1[c]	8.2 ± 0.1[d]	7.0 ± 0.1[c]	9.5 ± 0.1[e]	7.3 ± 0.1[c]

ND – not detected.

Values significantly different in the column are noted with different lower case letters [a-g]

Results in Table 4.12 show that the sterol composition ratios between the different oils were not significantly different ($p > 0.05$). For each sterol, there was no difference between flesh only oil and flesh and skin oil, although late season oil contains a higher proportion of β-sitosterol compared to early season oil and a lower proportion of campesterol and Δ5-Avenasterol. Moreover, the sterol profile conforms to the proposed Codex standard ranges, except for Δ5-Avenasterol which was higher than the recommended concentration range. (Codex Alimentarius Commission, 2021).

Table 4.13. Sterol concentration (mg kg^{-1} oil) in avocado oils at time zero (mean ± SEM, n=3).

	Flesh only mg kg^{-1} oil		Flesh and skin mg kg^{-1} oil		Whole fruit mg kg^{-1} oil	
	early	late	early	late	early	late
Campesterol	324 ± 3	243 ± 1	362 ± 3	283 ± 6	410 ± 8	262 ± 2
Stigmasterol	ND	ND	ND	ND	ND	ND
β-sitosterol	4185 ± 92	4790 ± 34	5183 ± 21	4947 ± 107	5568 ± 127	5522 ± 12
Δ5-Avenasterol	425 ± 12	461 ± 6	426 ± 2	429 ± 10	628 ± 16	438 ± 3
Total sterols	4935 ± 107	5613 ± 40	5871 ± 23	5618 ± 122	6607 ± 150	6243 ± 15

ND – not detected.

Table 4.13 shows the concentrations of the four identified sterols in the oil. Compared to flesh only oil, there are no significant differences in the concentrations of various sterols in flesh and skin oil ($p > 0.05$), except that early season flesh and skin oil contains a not significant higher content of β-sitosterol ($p > 0.05$). Whole fruit oil contains a significantly higher content of β-sitosterol compared to flesh only oil ($p < 0.05$), regardless of the season. Additionally, early season whole fruit oil has a higher concentration of Δ5-avenasterol compared to early season flesh only oil. The total sterol content of avocado oil significantly increased after the addition of skin and seed (($p<0.5$). The whole fruit oil had significantly higher total sterol content than both flesh and skin oil and flesh only oil ($p < 0.05$). Therefore, it was concluded that adding skin and seed during the malaxation process results in higher concentrations of sterols in the avocado oil.

4.8 Conclusions for chapter

In summary, although oil from whole fruit resulted in a lower oil yield (on a per volume basis) compared to the flesh and skin and flesh only oil, the oil had higher concentrations of carotenoids, chlorophylls, phenolics and sterols. Meanwhile, after adding skin and seed, the initial peroxide value and K values of avocado oil increased, despite no significant changes in free fatty acids. The oil from flesh and skin did not show significant differences ($p < 0.05$) in terms of carotenoids and total chlorophylls content from the flesh only oil, although it contained higher levels of pheophytin *a* and lower levels of chlorophyll *a*. The addition of skin did not have a significant impact on the increase in pigments in avocado oil. This could be attributed to the fact that the avocados used in the experiment were fully ripe with black colour skin for late season extraction. The content and variety of phenolics in the oil increased after the addition of skin and seed, indicating that the phenolic compounds from the skin and seed were soluble in the oil during extraction.

Chapter 5: Quality and storage stability of avocado oil with different treatments

Notable compositional changes were observed in avocado oil extracted with the addition of skin and seed as reported in Chapter 4. These changes could have significant effects on the stability and quality of the avocado oil over time. Therefore, testing parameters related to the quality of avocado oil and a storage trial was conducted to assess any differences in stability. Among the most commonly used quality parameters are the measurement of peroxide value and free fatty acids, which provide insights into the oxidation products and hydrolysis of fats. Ultraviolet spectrophotometric analysis at 232 and 270 nm also offers simple and valuable parameters for assessing the oxidative status of the oil.

Given the substantial increase in the content of phenolic compounds and pigments after adding skin and seed, changes in concentration of total phenolics, total chlorophyll, and pyropheophytin during storage were monitored. Oxidative stability using the Rancimat analysis was also used to determine the oxidative stability of the oil. Additionally, the colour and bitterness of the oil are quantitatively measured.

5.1 Peroxide value

The primary factor leading to the deterioration of avocado oil during storage is the development of oxidative rancidity (Flores *et al.*, 2019; Werman & Neeman, 1986). To assess the initial phase of oxidation in avocado oil, the extent of oxidation has often been gauged through the measurement of peroxide value (PV) (Gotoh & Wada, 2006; Zhang *et al.*, 2021). In the storage trial, avocado oils were stored in light-protected environments at temperatures of 20, 30, and 40°C and the change in PV over time is shown in Figure 5.1. Oils extracted from early season fruit were only stored at 30°C for all treatments but only at 20°C for flesh only, as less oil was able to be extracted from early season fruit. For late season fruit, oils extracted from all treatments were stored at 20, 30 and 40°C.

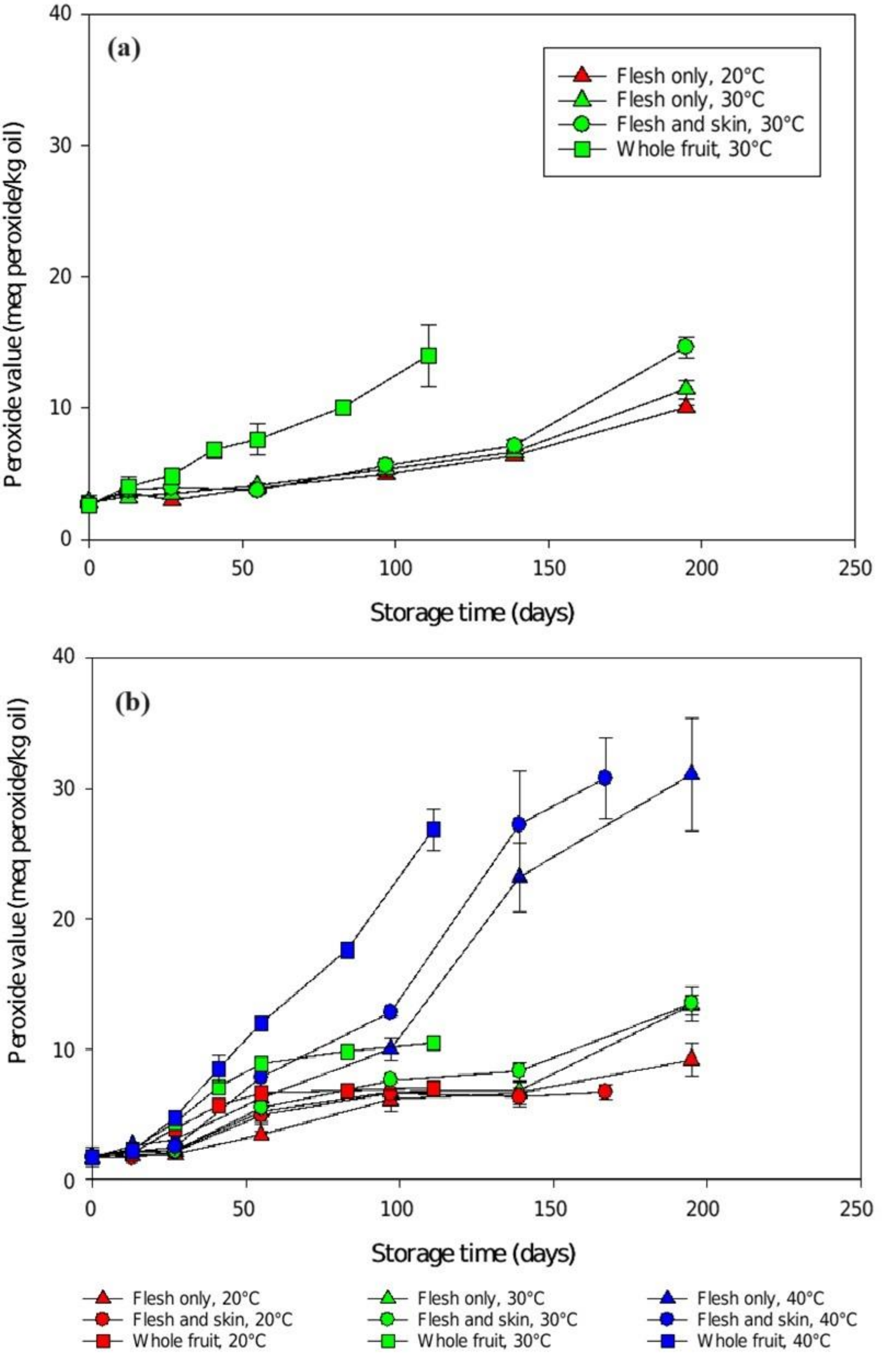


Figure 5.1. PV changes of (a) early season and (b) late season avocado oil with three extraction treatments stored at 20°C, 30°C and 40°C (mean ± SEM n=3).

Avocado oils from the early season had higher peroxide values at time zero, with 2.7 ± 0.2 meq kg^{-1} compared to 1.7 ± 0.1 meq kg^{-1} for late season oils. The rate of increase in PV for both flesh only oil and flesh and skin oil were nearly identical (Figure 5.1), but the increase in PV for whole fruit oil was faster than the other two treatments at the same storage temperature. It was also noteworthy that temperature resulted in a significant increase in PV in all treatments. At the storage temperature of 20°C, there was no significant difference in the increase of PV for all three oils. However, at 30°C, the rate of oxidation increased for all three treatments, with the increase in PV being slightly more pronounced for whole fruit oil compared to the other two oils. When oils were stored at 40°C, all three oils exhibited a much more rapid increase in PV and the final value at 195 days was significantly different from time zero ($p < 0.05$). For all temperatures the PV was higher for whole fruit over the storage period followed by flesh and skin oil, and lastly, flesh only oil. When comparing different treatments, the addition of skin and seed resulted in an oil that oxidised faster as a faster increase in PV was observed (Figure 5.1(b)). It can be concluded that higher storage temperatures accelerate the oxidation of avocado oil (Clodoveo *et al.*, 2007; Sanmartin *et al.*, 2018).

As reported in Chapter 4, the oil from whole fruit contained significantly more carotenoids, chlorophylls and phenolics, which appears to have an impact on the stability of the oil. Carotenoids are known to be prooxidant and chlorophyll can act as a photosensitizer for formation of O_2 in oil system, therefore their presence may be leading to a less stable oil which could then oxidise at a faster rate at elevated temperatures (Subagio & Morita, 2003; Usuki *et al.*, 1984). Therefore, a lower storage temperature is preferable, with a maximum of 30°C. Storing at 40°C is detrimental to the oil's quality and stability.

5.1.1 Evaluation of rate of peroxide value formation

The order of reaction and rate constant of peroxide value was determined to quantify the reaction rate of different samples during storage (Table 5.1). It was found that PV typically followed zero-order reaction kinetics in different oils and storage conditions and the reaction rate constant k increased with temperature, which indicated that primary oxidation in avocado oil accelerated with increasing storage temperature. These rate constants agreed with what was observed in Figure 5.1.

Table 5.1 Rate constant and order of reaction for peroxide value during storage

	Harvest time	Storage temperature (°C)	Rate constant k (meq kg^{-1} s^{-1})	R^2	Apparent reaction order
Flesh only	Early season	20	0.0339	0.925	0
	Early season	30	0.0399	0.917	0
	Late season	20	0.0406	0.978	0
	Late season	30	0.0552	0.926	0
	Late season	40	0.157	0.955	0
Flesh and skin	Early season	30	0.0521	0.839	0
	Late season	20	0.0341	0.858	0
	Late season	30	0.0592	0.965	0
	Late season	40	0.185	0.960	0
Whole Fruit	Early season	30	0.0991	0.989	0
	Late season	20	0.0523	0.813	0
	Late season	30	0.0866	0.890	0
	Late season	40	0.231	0.980	0

5.2 Free fatty acids

Free fatty acids (FFA) result from the hydrolysis of lipids, generated through chemical or enzymatic processes. The formation of FFA accelerates oxidative reactions, thereby reducing the shelf life (Frega *et al.*, 1999; Miyashita & Takagi, 1986). This occurrence is especially noteworthy in water-containing lipidic matrices. The production of FFA necessitates water as a reactant.

Compared to the initial FFA values shown in Section 4.1.4, it was found that the FFA values of different oils from each treatment and from early or late season fruit remained relatively constant and statistically unchanged at various storage temperatures ($p > 0.05$), all at approximately 0.2% to 0.3%, which means there were no hydrolysis reactions during storage (Figure 5.2). This stability was attributed to the fact that avocado oils were centrifuged to remove water. Moreover, the oils were nitrogen flushed before storage and shielded from light, which eliminated the conditions conducive to lipid hydrolysis. This emphasized the importance of water-free production conditions and light reduced storage in enhancing the oxidative stability of avocado oil.

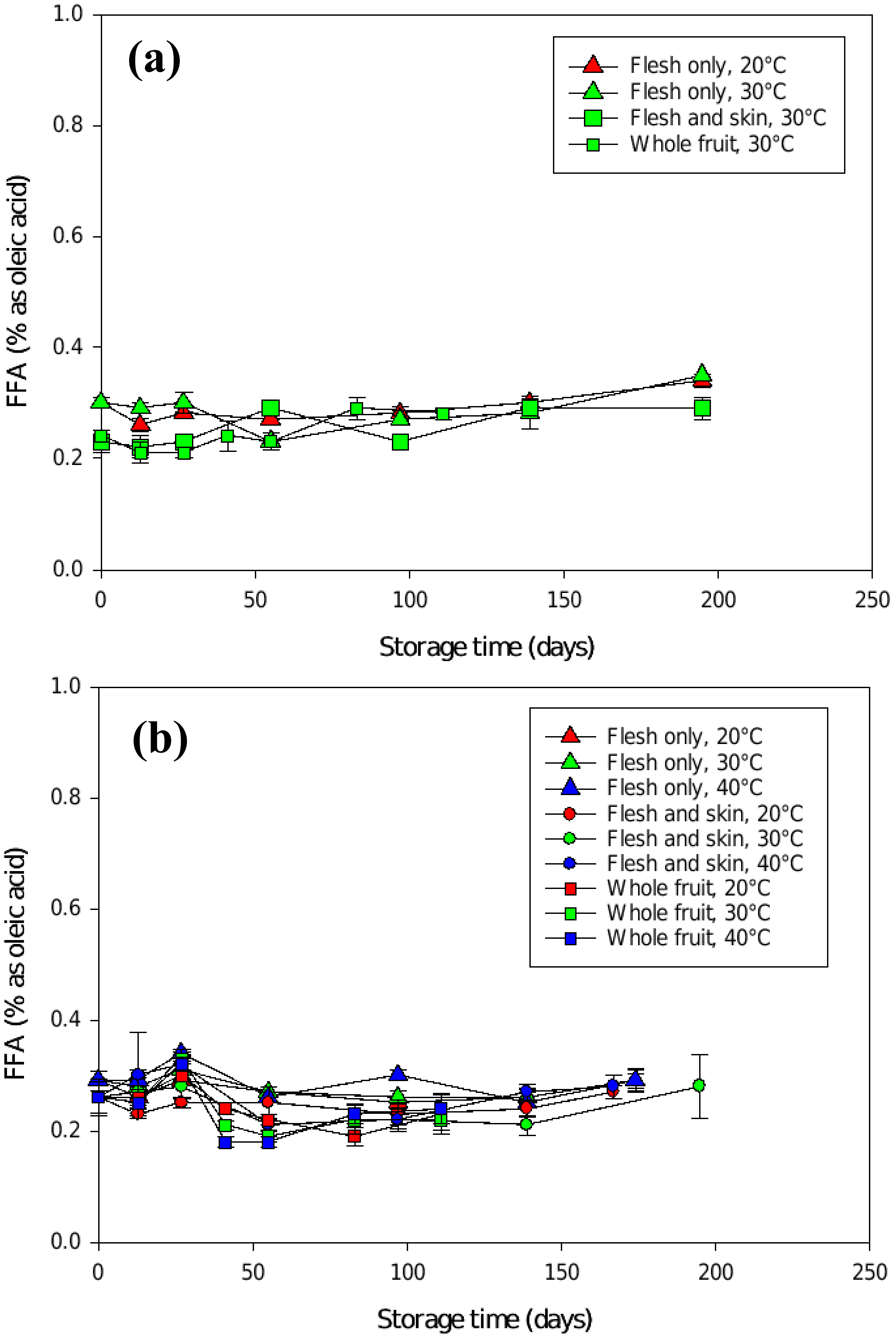


Figure 5.2. FFA changes of (a) early season and (b) late season avocado oil extracted with three treatments stored at 20°C, 30°C and 40°C (mean ± SEM n=3).

5.3 Specific extinction coefficient K_{232}, K_{270}

As mentioned in Section 4.1.4, K_{232} is related to the primary oxidation products, which can reflect the degree of oxidation in oil. From Figure 5.3 in can be seen that, the K_{232} value for late season oils was higher than that of early season oils for all treatments at time zero, they all increased at a similar rate at the same temperature. They exhibited slow increases at 20°C and 30°C, but a rapid rise at 40°C. The K_{232} rate of increase for whole fruit oil was faster than the other two oils, regardless of the season, even though this oil had higher K_{232} values at time zero. This indicated a significant positive correlation between temperature and the primary oxidation of the oil, with whole fruit oil undergoing primary oxidation more rapidly. These results align with the trend observed with PV, confirming that K232 can reflect the extent of primary oxidation in avocado oil.

K_{270} represented the secondary oxidation products, for example carbonyls, trienes and aldehydes (Elez-Martinez *et al.*, 2005). The variation of K_{270} values is shown in Figure 5.4. Under storage at 20°C and 30°C, the increase in the K_{270} values for all oils were not significant ($p > 0.05$), no matter early season or late season. However, the K_{270} value of whole fruit oil was much higher than the other two oils. When the oils were stored at 40°C, the K_{270} values showed a significant increase for all treatments ($p < 0.05$), with whole fruit oil exhibiting the most substantial variation. Therefore, adding skin did not have a significant impact on the secondary oxidation of oil. However, adding seed increased the initial secondary oxidation products and accelerated their formation during storage. Additionally, temperature had a significant impact on the formation of secondary oxidation products during storage. This can be seen also with the reaction rate constants at higher storage temperatures (Table 5.2)

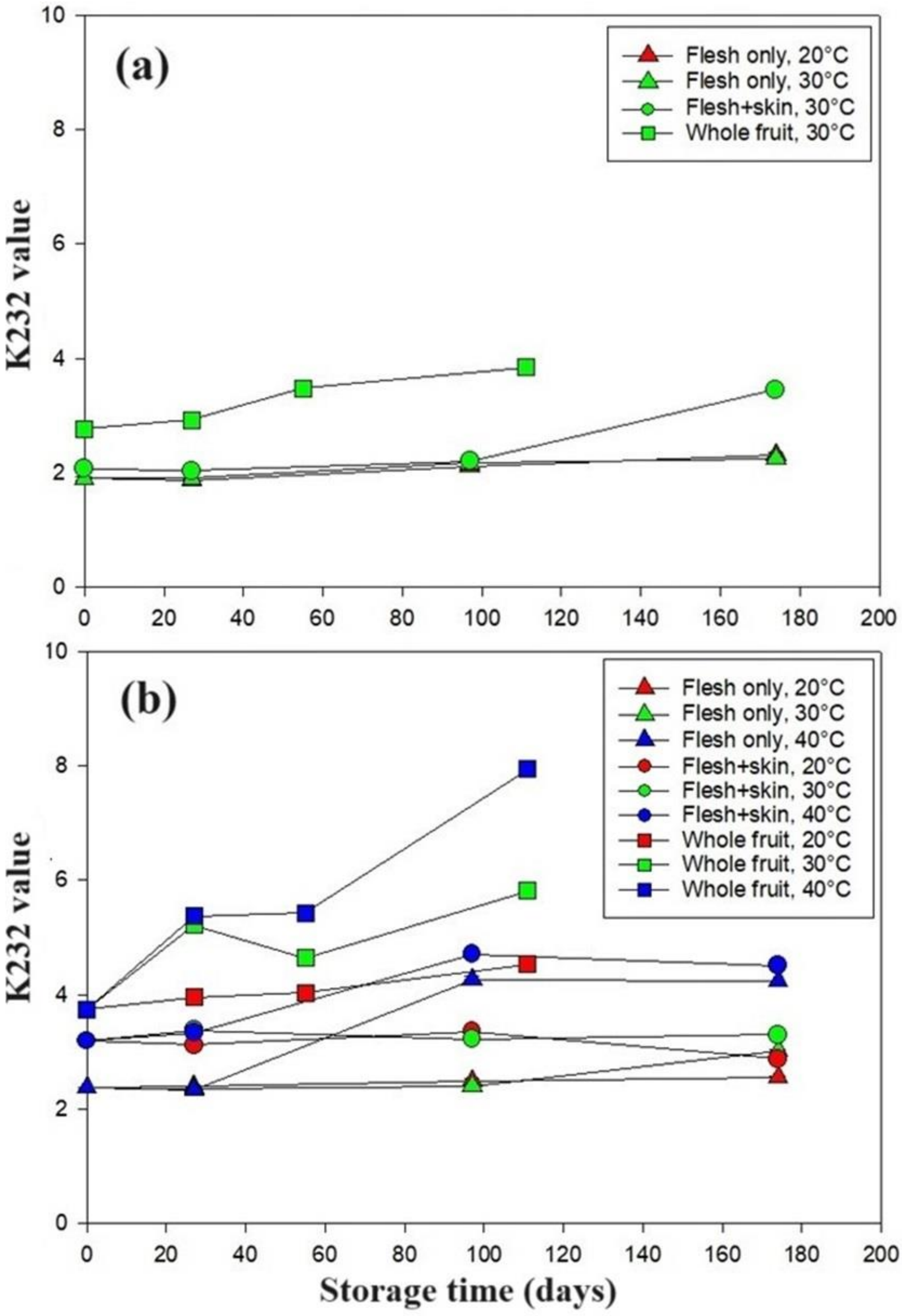


Figure 5.3. K_{232} value changes of (a) early season and (b) late season avocado oil with three treatments stored at 20°C, 30°C and 40 °C (mean ± SEM n=3).

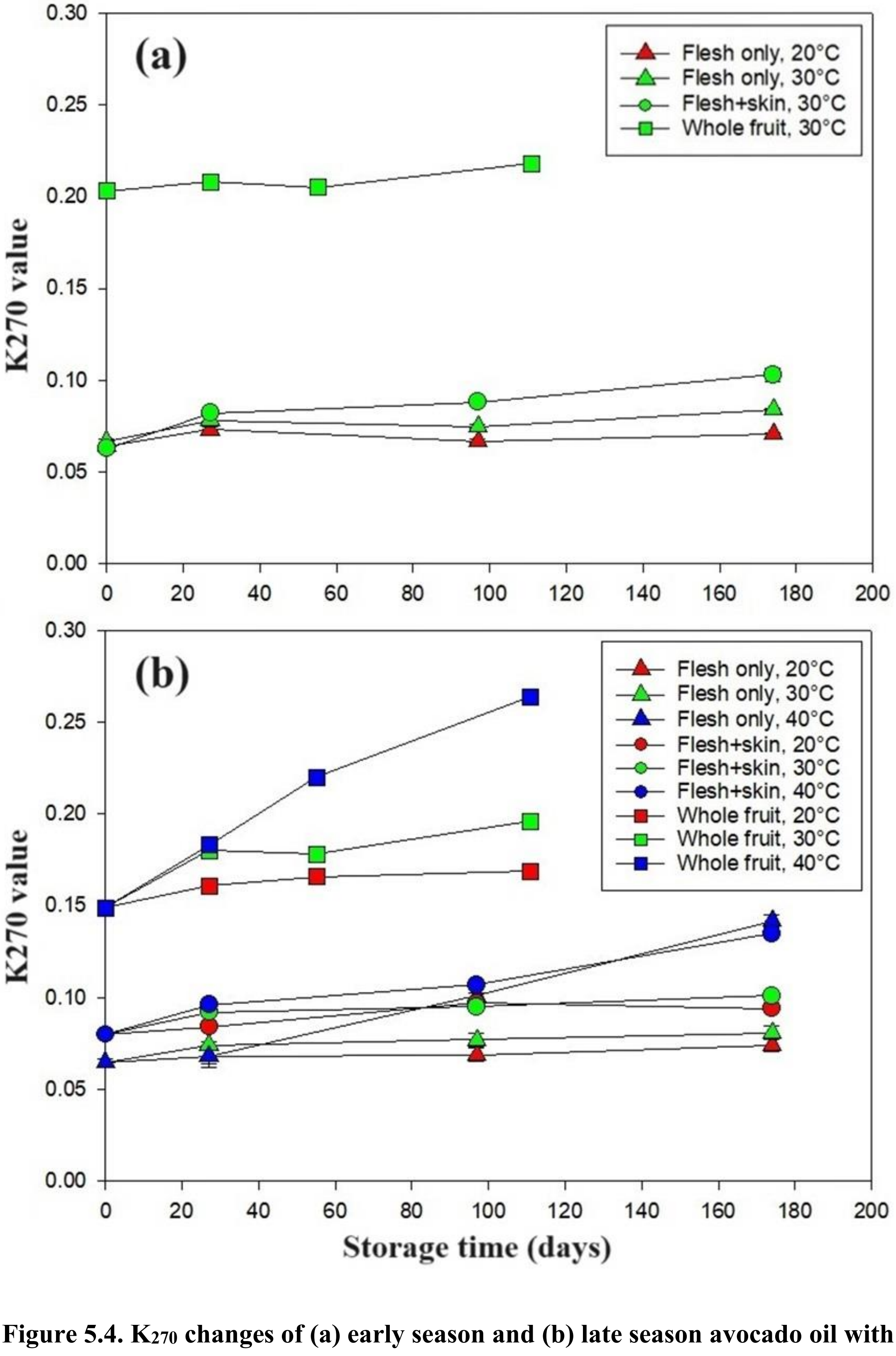


Figure 5.4. K_{270} changes of (a) early season and (b) late season avocado oil with three treatments stored at 20°C, 30°C and 40°C (mean ± SEM n=3).

5.3.1 Evaluation of rate of reaction for K_{232} and K_{270}

The order of reaction and rate constant of K_{232} and K_{270} was determined to quantify the reaction rate of different samples during storage (Tables 5.2 and 5.3). For K_{232} the order of reaction was found to best fit zero order, while for K_{270} indicating secondary oxidation, this was found to follow first order kinetics. The reaction rate constant increased with temperature for K_{232}, which indicated that primary oxidation in avocado oil accelerated with increasing storage temperature. These rate constants agreed with what was observed in Figure 5.3 and 5.4.

Table 5.2 Rate constant and order of reaction for K_{232} during storage

	Harvest time	**Storage temperature (°C)**	**Rate constant *k* (s^{-1})**	**R^2**	**Apparent reaction order**
Flesh only	Early season	20	0.0022	0.922	0
	Early season	30	0.0026	0.953	0
	Late season	20	0.0011	0.990	0
	Late season	30	0.0036	0.758	0
	Late season	40	0.0126	0.804	0
Flesh and skin	Early season	30	0.0078	0.804	0
	Late season	20	0.0041	0.887	0
	Late season	30	0.0007	0.917	0
	Late season	40	0.0087	0.749	0
Whole Fruit	Early season	30	0.0102	0.944	0
	Late season	20	0.0069	0.974	0
	Late season	30	0.0187	0.995	0
	Late season	40	0.0356	0.946	0

Table 5.3 Rate constant and order of reaction for K_{270} during storage

	Harvest time	**Storage temperature (°C)**	**Rate constant *k* (s^{-1})**	**R^2**	**Apparent reaction order**
Flesh only	Early season	20	0.0006	0.894	1
	Early season	30	0.0013	0.996	1
	Late season	20	0.0006	0.873	1
	Late season	30	0.0011	0.807	1
	Late season	40	0.0047	0.991	1
Flesh and skin	Early season	30	0.0028	0.976	1
	Late season	20	0.0010	0.729	1
	Late season	30	0.0011	0.773	1
	Late season	40	0.0028	0.951	1
Whole Fruit	Early season	30	0.0006	0.962	1
	Late season	20	0.0010	0.793	1
	Late season	30	0.0021	0.769	1
	Late season	40	0.0050	0.957	1

5.4 Oxidative Stability with Rancimat analysis

The Rancimat analysis is an accelerated method designed for evaluating the oxidative stability of edible fats and oils. This test determines the induction time, which signifies the duration before the onset of rapid degradation of the oil (Farhoosh *et al.*, 2008). Oils were heated and oxidized at 110°C, then volatile secondary byproducts produced during these reactions are released and transported into the measurement vessel through the airflow (Section 3.6.9). The induction time of each sample represents its oxidative stability, indicating the resistance to oxidation (Figure 5.5).

The induction time for each treatment in early season oil was slightly higher than the induction times for all treatments for late season oils (Table 5.4). Moreover, the addition of skin and seed significantly increases the induction time for both early and late season oils. Thus, the Rancimat analysis shows that the addition of skin and seed improved oil stability.

Table 5.4. Induction time of avocado oil with three treatments (mean ± SEM n=3).

	Induction time (hours)		
	Flesh only	**Flesh and skin**	**Whole fruit**
Early season	9.39 ± 0.38	15.43 ± 0.53	13.99 ± 0.15
Late season	8.82 ± 0.46	13.16 ± 0.17	12.51± 0.16

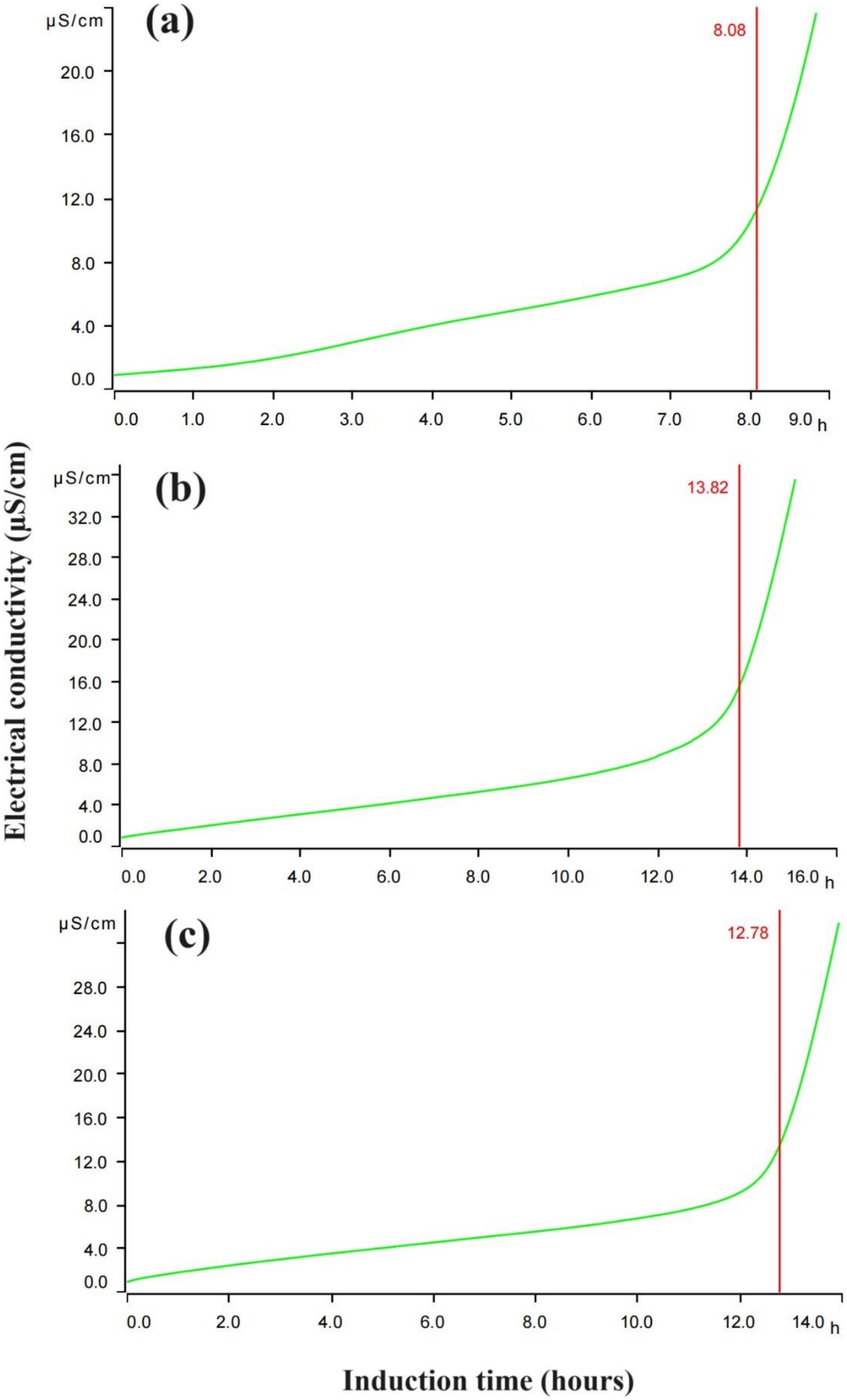


Figure 5.5. Representative Rancimat result report of avocado oil, (a) Flesh only, (b) Flesh and skin, (c) Whole fruit.

5.5 Total phenolics

The changes in total phenolics in the three treatments of late season fruit oil stored at different temperatures are shown in Figure 5.6. The total phenolic content of all samples decreased significantly compared to the time zero values ($p < 0.05$). Moreover, the trend of total phenolic content for all three treatments showed a more significant decline at higher temperatures ($p < 0.05$) no matter what season. Even though the whole fruit oil had the highest total phenolic content, its declining trend was the most significantly influenced by temperature amongst the three treatments.

As the quality and stability of oil are affected by the total phenolic content which act as antioxidants (Tan *et al.*, 2018a), the decreasing phenolic content means phenolic compounds were degrading during storage. This may be caused by the interaction with other components in avocado oil or phenolics are used up as antioxidants. It can also be concluded that higher storage temperatures significantly impacted the degradation of phenolic compounds.

Table 5.5 Rate constant and order of reaction for total phenolics during storage

	Harvest time	Storage temperature (°C)	Rate constant *k* (s^{-1})	R^2	Apparent reaction order
Flesh only	Early season	20	0.0032	0.883	1
	Early season	30	0.0048	0.994	1
	Late season	20	0.0015	0.816	1
	Late season	30	0.0013	0.739	1
	Late season	40	0.0032	0.885	1
Flesh and skin	Early season	30	0.0011	0.801	1
	Late season	20	0.0019	0.855	1
	Late season	30	0.0018	0.906	1
	Late season	40	0.0021	0.967	1
Whole Fruit	Early season	30	0.0022	0.924	1
	Late season	20	0.0020	0.972	1
	Late season	30	0.0033	0.768	1
	Late season	40	0.0055	0.842	1

Table 5.5 shows the reaction rate constants for the loss of phenolics during the storage period. The rate of loss of phenolics was greater at the higher temperatures as mentioned above. The loss of phenolics was found to follow first order kinetics. Regardless of the season, the whole fruit oil had the highest starting value of total phenolic, followed by flesh+skin oil and the flesh only oil had the lowest content They have different starting

values as the oils extracted more phenolics from skin and seed, which may add to higher initial bitterness values.

5.6 Bitterness

Bitterness in oil is commonly regarded as a favourable sensory characteristic in olive oil that elevates the overall flavour profile (Inarejos-Garcia *et al.*, 2009; Mateos *et al.*, 2004). However, its impact in avocado oil is currently inconclusive, necessitating further sensory tests to determine its effect. Bitterness is a quality is frequently associated with the presence of phenolic compounds in edible oils (Favati *et al.*, 2013; Siliani *et al.*, 2006). Sensory testing was not carried in this project as not enough oil was able to be extracted for each treatment as only 6-8 fruits were used for each malaxer.

Bitterness variations in avocado oil are illustrated in Figure 5.7. It was observed that although the bitterness did not significantly increase after adding skin tissue ($p > 0.05$), the bitterness of the oil significantly increased after adding seed tissue ($p < 0.05$). During the storage, the bitterness of flesh only and flesh and skin oil did not show significant increase at all storage temperatures ($p > 0.05$). However, in the case of whole fruit oil, bitterness increased with prolonged storage time and increasing the storage temperature accelerated this process. This correlation may be attributed to the higher phenolic content in whole fruit oil and the phenolics present reacting to form polymerised phenolics, for example tannins as polymerised phenolics are reported to be more bitter (Inarejos-Garcia *et al.*, 2009). Similar to the trend observed in the total phenolics, the phenolic compounds underwent significant reduction with the passage of storage time ($p < 0.05$), consequently contributing to substantial alterations in the bitterness of the oil.

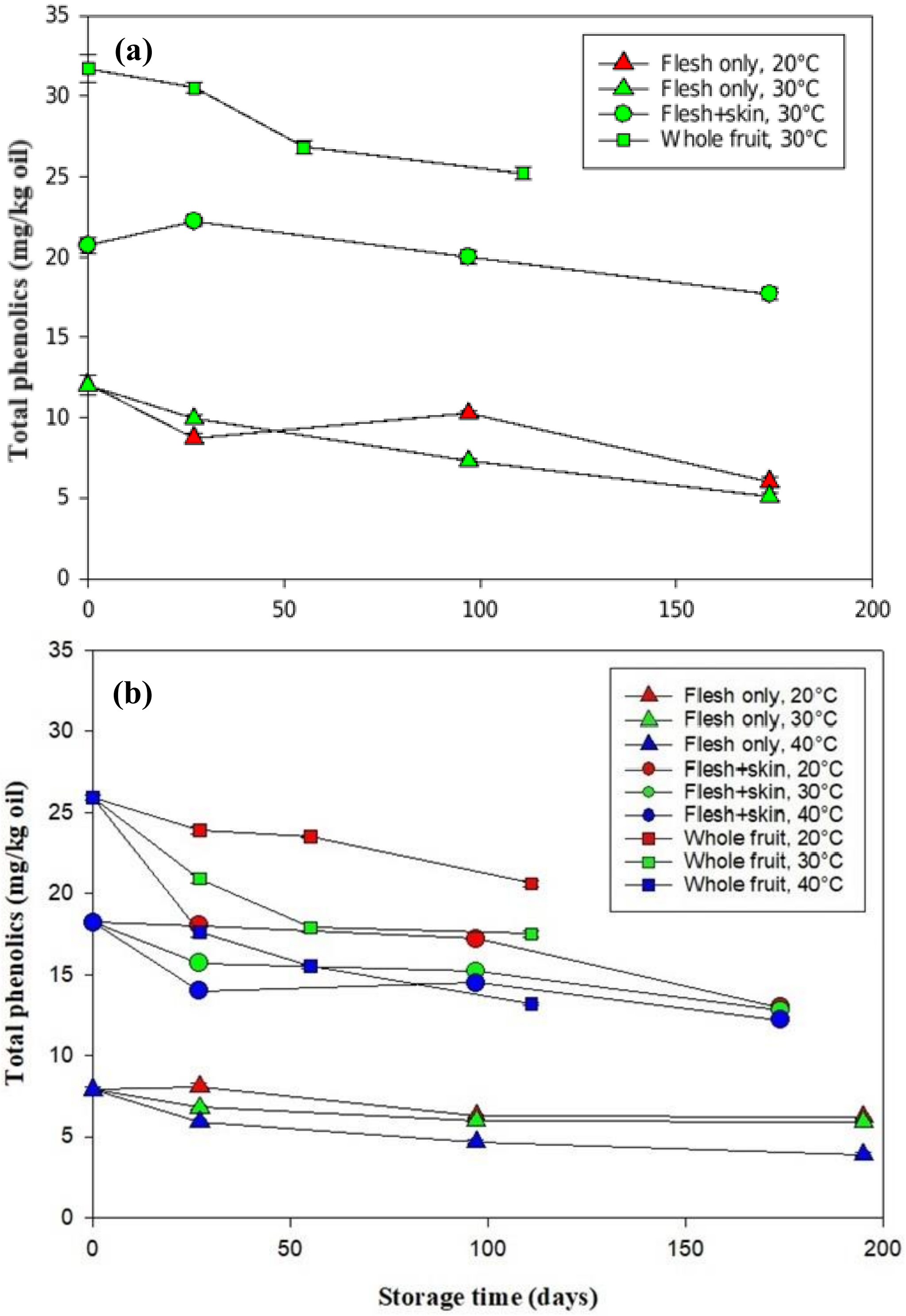


Figure 5.6. Total phenolics changes of (a) early season and (b) late season avocado oil with three treatments stored at 20°C, 30°C and 40°C (mean ± SEM n=3).

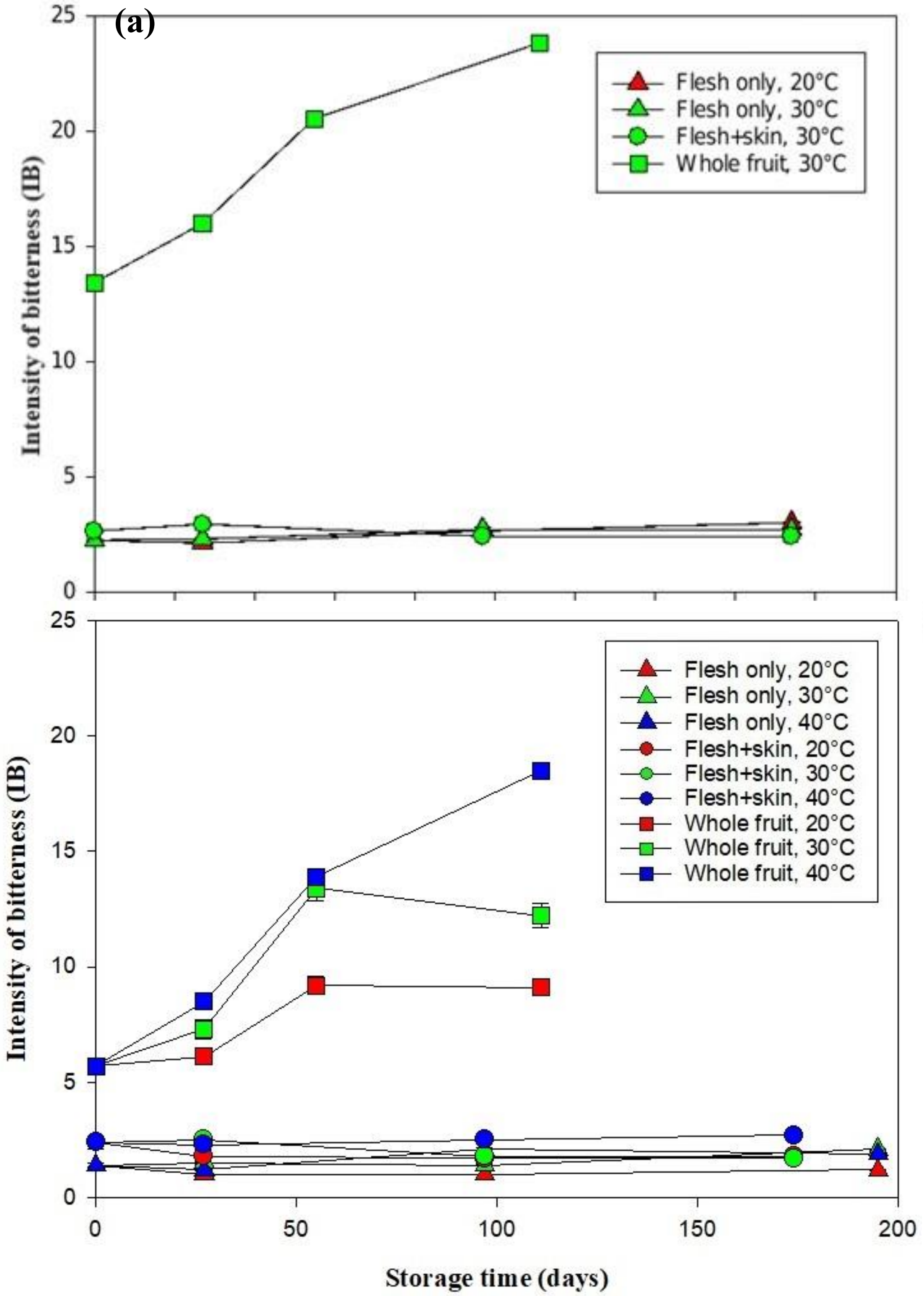


Figure 5.7. Intensity of bitterness changes of (a) early season and (b) late season avocado oil with three treatments stored at 20°C, 30°C and 40°C (mean ± SEM n=3).

5.7 Total chlorophyll

Chlorophyll's antioxidant properties contribute to neutralizing damage caused by free radicals in the body. It aids in promoting the immune system and effectively prevents infections (Martins *et al.*, 2023; Minguez-Mosquera *et al.*, 2008). Previous research has reported the prooxidant activities of chlorophylls and pheophytins on oil (Choe *et al.*, 2019; Park *et al.*, 2013; Usuki *et al.*, 1984; Zeb & Murkovic, 2013). Simultaneously, serving as a natural pigment, it can have a negative impact on the storage of avocado, although it contributes to visual appeal.

The trend of total chlorophyll changes in stored avocado oils is shown in Figure 5.8. Total chlorophyll content was significantly influenced by temperature and oil composition ($p < 0.05$). At storage temperatures of 20°C and 30°C, the total chlorophyll content of flesh only and flesh and skin oil remain mostly unchanged ($p > 0.05$). However, at 40°C, there is an accelerated decline in the total chlorophyll content. Whole fruit oil was most affected by temperature, as the decline in chlorophyll content increased significantly with the rise in storage temperature ($p < 0.05$). This may be attributed to the higher total chlorophyll content in whole fruit oil, making it more susceptible to temperature effects. It was found earlier that the addition of avocado skin and seed accelerated the oxidation process, especially at higher temperatures, which corresponded to also higher concentrations of pigments. The decreasing trend in total chlorophyll is similar to that of total phenolic content but opposite to that of PV. Oils with higher total chlorophyll content exhibit a faster increase in PV, while the decline in total phenolic content is more rapid. This may indicate the chlorophylls in avocado oil accelerate the oil oxidation and decomposition of phenolics.

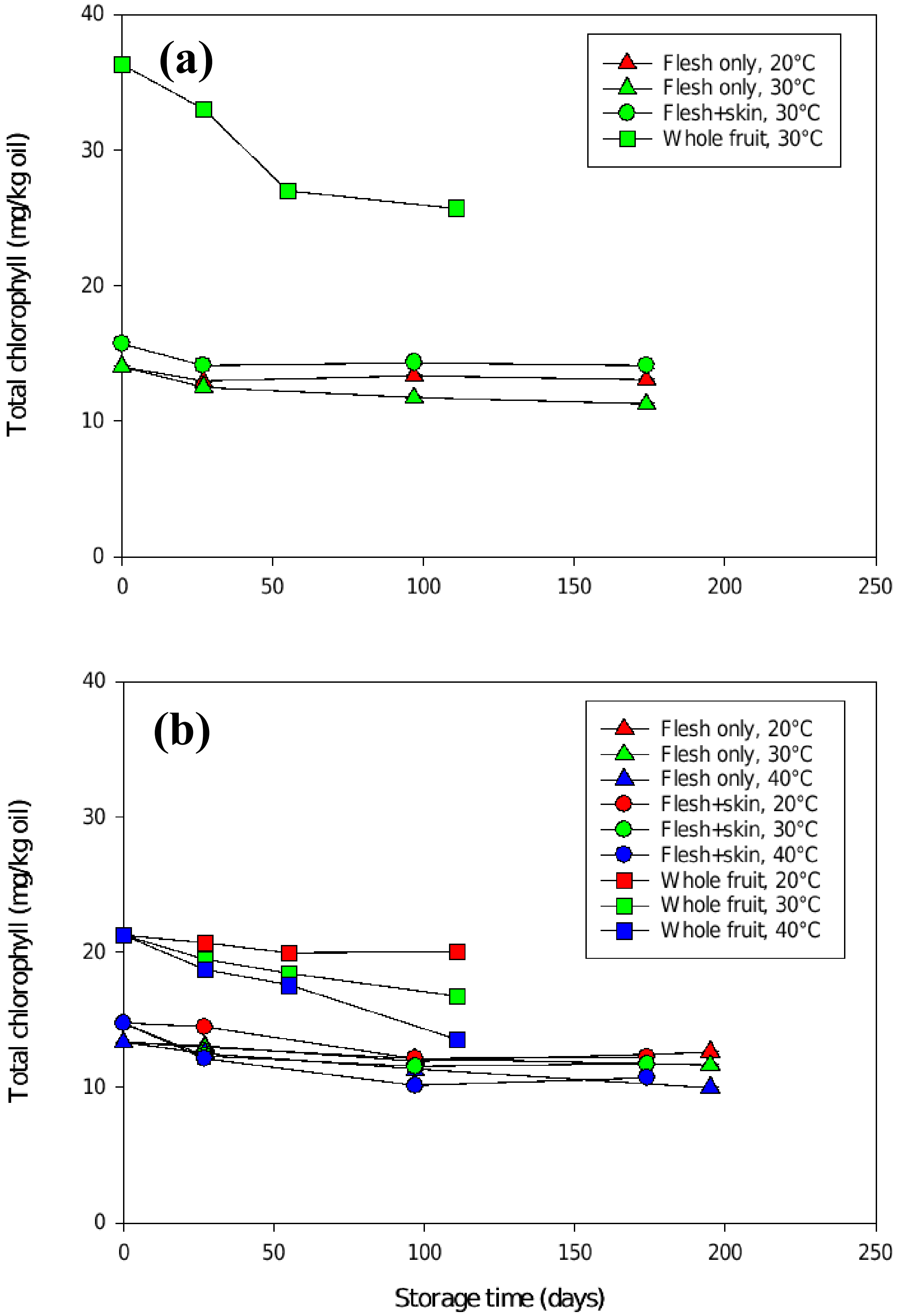


Figure 5.8. Change in total chlorophylls in oils extracted from (a) early season and (b) late season avocado oil with three treatments stored at 20°C, 30°C and 40°C

Table 5.6 shows the reaction rate constants for the loss of total chlorophyll during the storage period. The rate of loss of total chlorophyll was greater at the higher temperatures as mentioned above. The loss of phenolics was found to follow second order kinetics.

Table 5.6 Rate constant and order of reaction for total chlorophyll during storage

	Harvest time	Storage temperature (°C)	Rate constant k (kg mg^{-1} s^{-1})	R^2	Apparent reaction order
Flesh only	Early season	20	3.4×10^{-5}	0.955	2
	Early season	30	9.3×10^{-5}	0.870	2
	Late season	20	2.1×10^{-5}	0.909	2
	Late season	30	4.9×10^{-5}	0.956	2
	Late season	40	10.2×10^{-5}	0.999	2
Flesh and skin	Early season	30	4.3×10^{-5}	0.888	2
	Late season	20	8.8×10^{-5}	0.789	2
	Late season	30	9.0×10^{-5}	0.670	2
	Late season	40	20.4×10^{-5}	0.864	2
Whole Fruit	Early season	30	10.3×10^{-5}	0.876	2
	Late season	20	3.4×10^{-5}	0.824	2
	Late season	30	12.1×10^{-5}	0.987	2
	Late season	40	24.2×10^{-5}	0.978	2

5.8 Oil colour

The colour of avocado oil is influenced by carotenoid and chlorophyll pigments extracted from both the flesh and skin tissues during the extraction process. The storage of avocado oil leads to a reduction in these pigments dependent on factors such as temperature, light exposure, and oxygen levels (Wong *et al.*, 2008; Woolf *et al.*, 2009). Therefore, the colour of avocado oil serves as a crucial quality monitoring indicator during storage, concurrently reflecting the chlorophyll content in the oil. Lightness (L) and greenness (a^*) of avocado oil were measured during storage using a Minolta colorimeter (Section 3.6.5).

Figure 5.9 shows the L values (lightness) of late season oil for three treatments, arranged from dark to light as follows: whole fruit, flesh only, and then flesh and skin. This conclusion agrees with the avocado photos at time zero shown in Figure 4.2. Similarly, both early season oil and late season oil experienced a transition in lightness from darker shades to lighter shades during the storage process, but with no significant differences observed between the oil from the early vs late season ($p > 0.05$).

As seen in Figure 4.2, the green colour of the oil from whole fruit oil appeared to be more intense green than the other two treatments, consistent with the initial a* values shown in Figure 5.10. The variation in the greenness of avocado oil was notably influenced by temperature, as evidenced by the significant differences in greenness among the three treatments at 200 days ($p < 0.05$). Oil stored at 20°C exhibited higher greenness (lower a* values) compared to that stored at 30°C, while oil stored at 40°C was the least, indicating chlorophyll breakdown (section 5.7). This again confirms that the higher the temperature, the faster the degradation rate of chlorophyll.

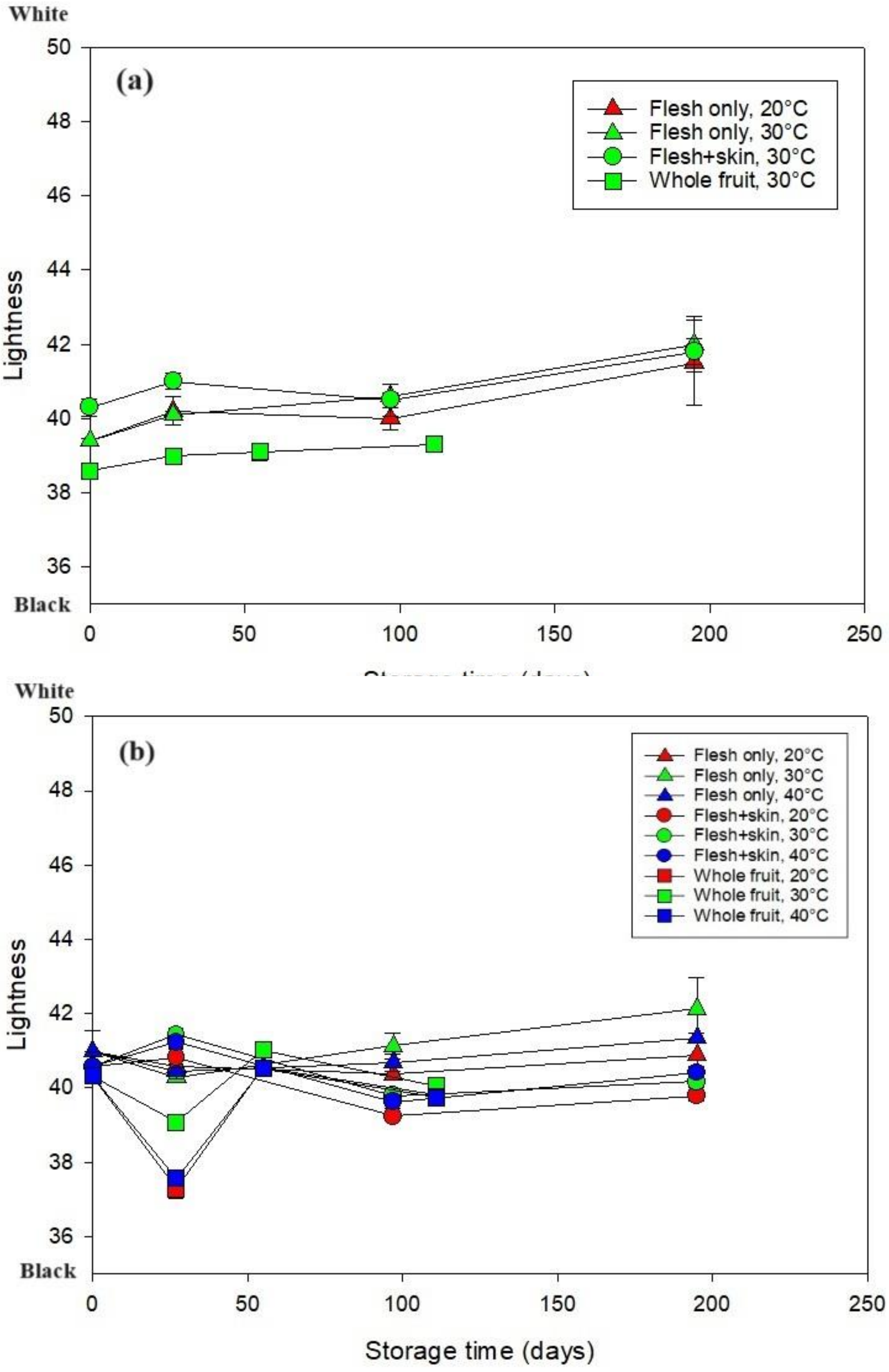
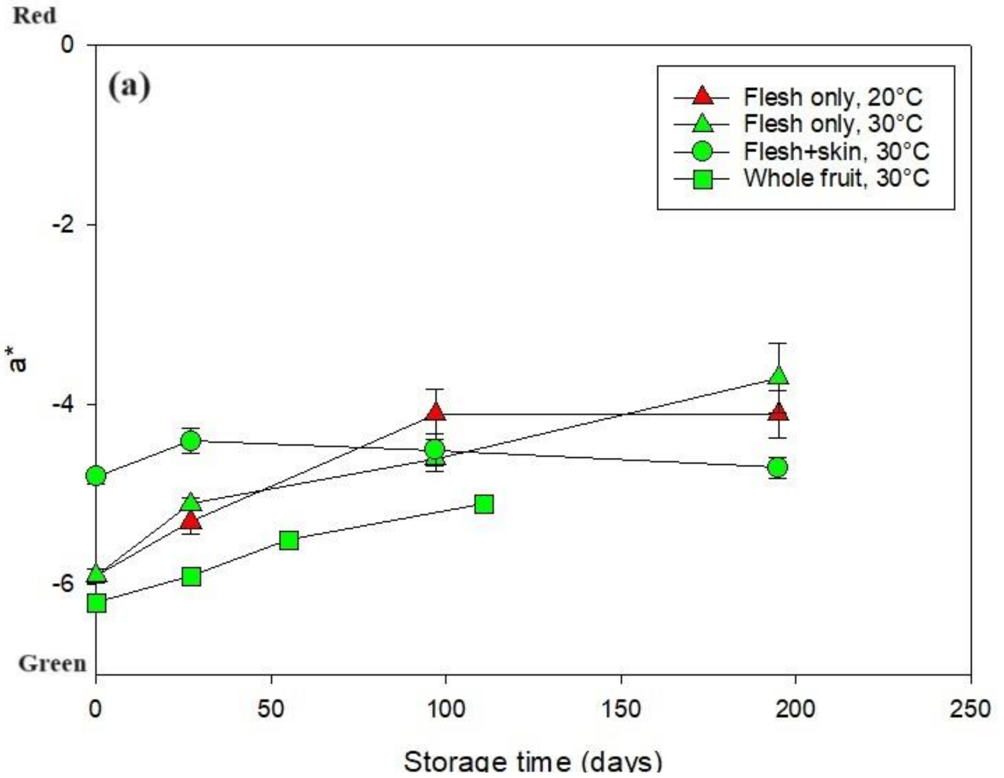


Figure 5.9. Changes in L values (Lightness) in oils extracted from (a) early season and (b) late season avocado oil with three treatments stored at 20°C, 30°C and 40°C (mean ± SEM n=3).



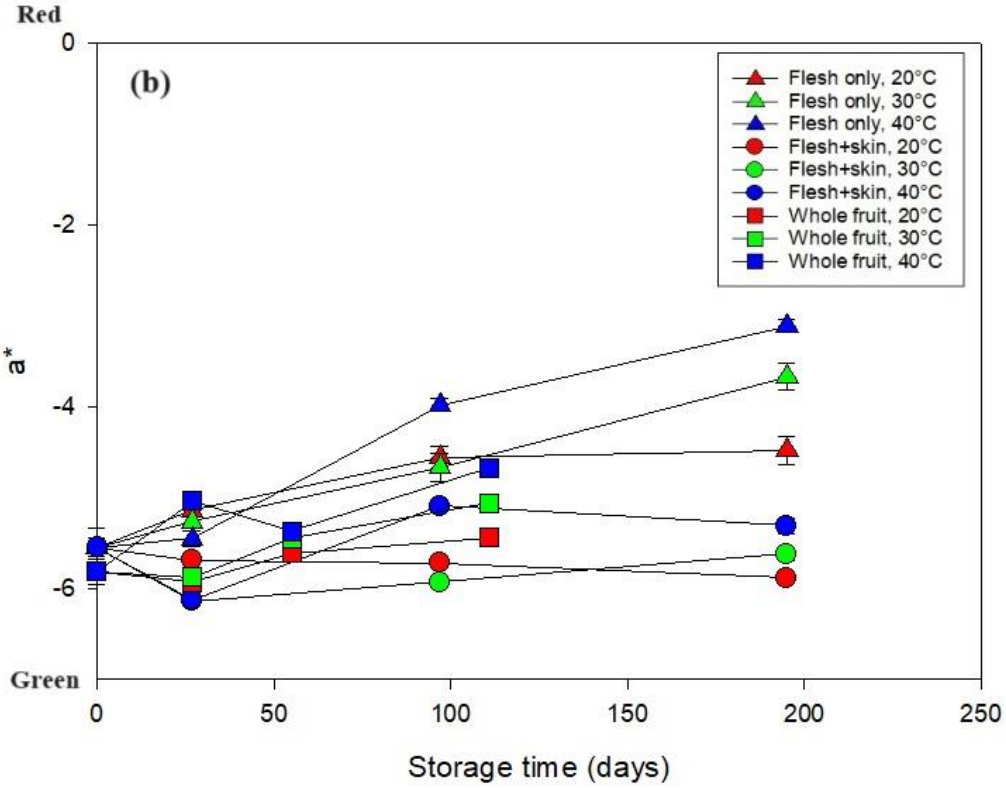


Figure 5.10. Changes in a* values (Green/Red colour) in oils extracted from (a) early season and (b) late season avocado oil with three treatments stored at 20°C, 30°C and 40°C (mean ± SEM n=3).

5.9 Pyropheophytins

Chlorophylls contribute to a prooxidant function in promoting the oxidation of avocado oil (Choe *et al.*, 2019; Park *et al.*, 2013). During the storage of avocado oil, chlorophyll degradation takes place, leading to the formation of pyropheophytin (Lanfer-Marquez *et al.*, 2005). This compound is not generated during the oil extraction process, thus serving as a valuable parameter for monitoring the loss and the deterioration of freshness during storage (Aparicio-Ruiz *et al.*, 2012). Figure 5.11 shows a representative HPLC chromatogram of pyropheophytin *a.* The three peaks labelled from left to right are pheophytin *a*, pheophytin *a'*, and pyropheophytin *a*.

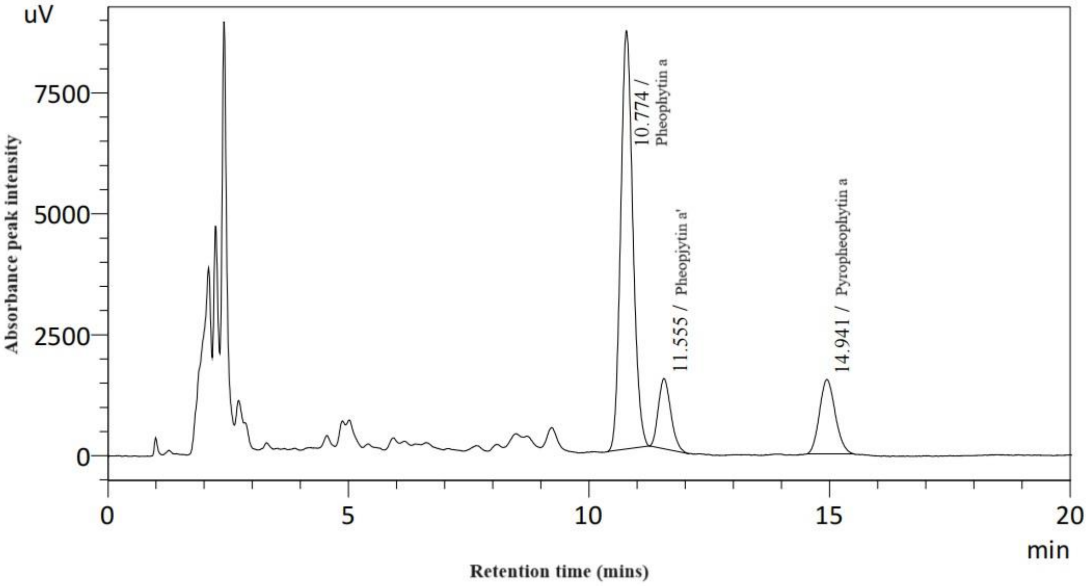


Figure 5.11. Representative HPLC chromatograms of pyropheophytin compounds in avocado oil

The mass percentage variation of pyropheophytin *a* during storage of oil is shown in Figure 5.12. The formation of pyropheophytin in the avocado oil was not significantly different between seasons ($p > 0.05$). The increase in pyropheophytin *a* was primarily influenced by temperature, as the percentage variation of pyropheophytins in oils with same treatments were similar. With the increase in temperature, the production rate of pyropheophytins rapidly increased. This may be due to the fact that higher temperatures can accelerate the breakdown of chlorophyll into pyropheophytin.

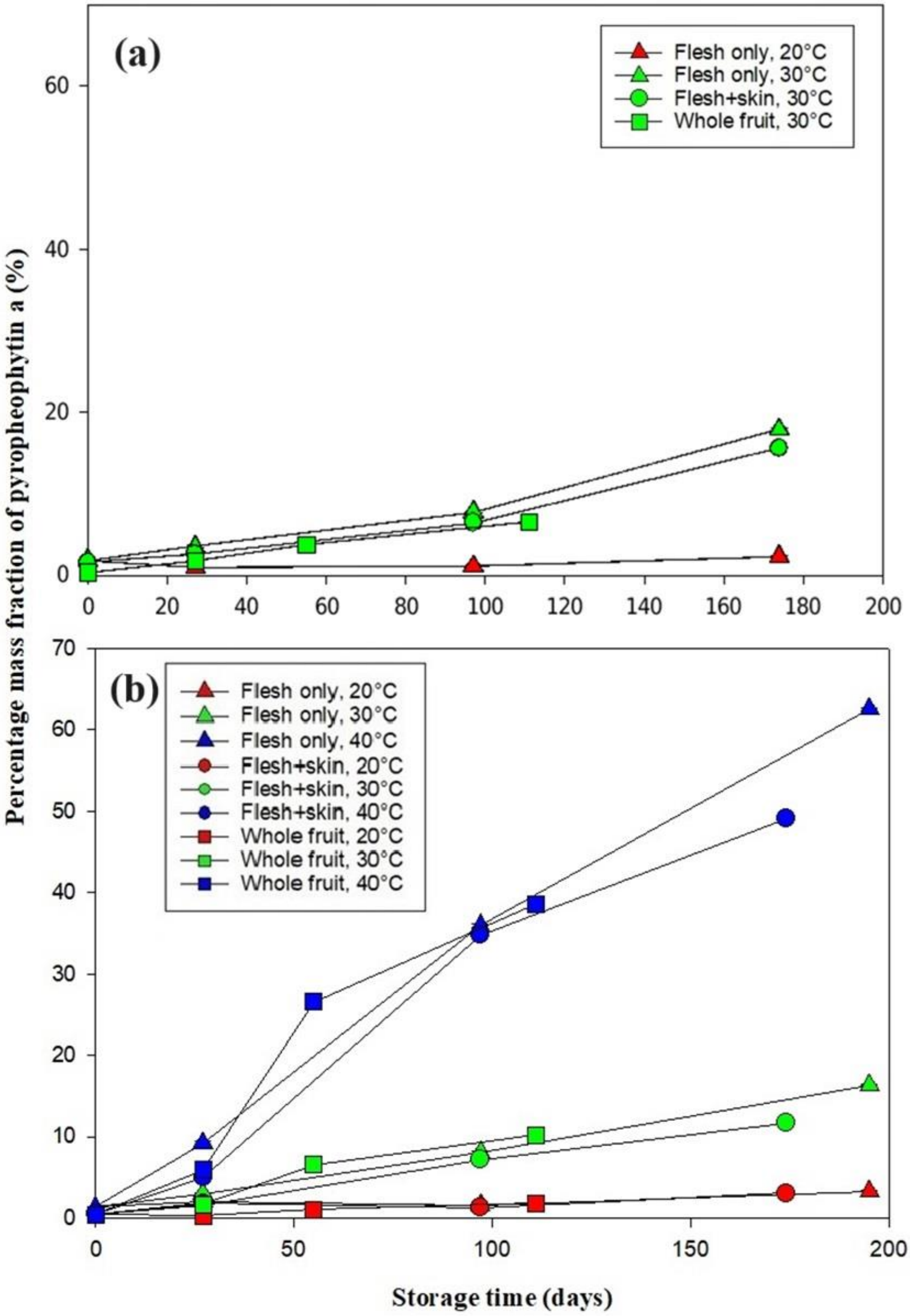


Figure 5.12. Change in percentage of pyropheophytins in oil extracted from (a) early season and (b) late season avocado oil with three treatments stored at 20°C, 30°C and 40°C (mean ± SEM n=3).

5.11 Conclusions for this chapter

During the storage of avocado oil, the PV and K values increased over time and higher temperatures accelerated the rate of this increase, which indicates that temperature promotes the oxidation of oil. Although the oxidative stability measured by Rancimat suggested improved stability with added skin and seed, the whole fruit oil oxidized the fastest (higher PV and K value, more rapid decline in phenolics and pigments), especially at higher temperatures. The FFA of all samples showed no significant changes during the storage process, regardless of the storage temperature. This indicates that hydrolysis of lipids in the oil did not occur. The total phenolic content of avocado oils decreased during storage, but the bitterness of oils increased for all samples, which may be cause by the decomposition of phenolics into larger phenolic compounds that did not absorb at 760 nm after treatment with the Folin Ciocalteau reagent. The greenness of avocado oil decreased with the increase in storage time, aligning with the trend found in total chlorophyll. After adding skin and seed, avocado oil contained higher levels of chlorophylls and phenolics yet oxidized more rapidly. This could be attributed to interactions between these components, which may enhance the rate of oxidation.

Chapter 6: Overall Discussion

The overall aim of this study was to investigate the impact of incorporating avocado skin and seed during the cold-pressing process of avocado oil on the oil yield, composition and stability of the oil. The extracted oil was analysed for its composition and quality indicators and the oils were stored for an extended period at elevated temperatures. Three different tissue combinations in the malaxers were investigated: flesh with 10% of the skin tissue (flesh only), flesh with all skin (flesh+skin), flesh with all skin and seed (whole fruit).

'Hass' avocados used in this study were harvested in two times in the season: late season (July 2022) and early season (August 2022). It was observed that the dry matter content of avocados in the early season (26.5% g/g wet tissue) was lower than that of avocados in the late season (33.8% g/g wet tissue), as expected as the dry matter will increase over the harvest season. Additionally, the total oil content of flesh in the early season (17.9% oil g/g wet tissue) was also lower compared to the late season (23.7% oil g/g wet tissue). This confirmed the positive correlation between the amount of dry matter and the oil content in avocados, consistent with previous research findings (Woolf *et al.*, 2009; Yang *et al.*, 2020). Similarly, the oil yield for all three treatments in the early season were lower than those in late season, correlating positively with the amount of dry matter. When comparing the three treatments, the oil extraction volume was highest for the flesh only oil, followed by the flesh and skin oil, and the lowest for the whole fruit oil. The flesh contains the most oil recoverable, up to 25% oil and an avocado on average contains approximately 68% flesh (Woolf *et al.*, 2009). Less oil extracted with flesh+skin and whole fruit was attributed to the reduced content of flesh per malaxer due to the addition of skin and seed. Therefore, adding skin and seed during malaxing reduces the overall oil yield of avocado oil per malaxer. Noting that avocado fruits with higher maturity would still give a higher oil yield.

PV and FFA are considered to be the important quality parameters for extra virgin oils (International Olive Council, 2019b) . If the FFA% is less than 0.5% and the PV is less than 4.0 meq/kg oil, it can be regarded as extra virgin avocado oil (Woolf *et al.*, 2009). This study has also measured the UV absorbance value K_{232} and K_{270} for detecting the primary and secondary oxidation products in the oil, respectively (Angerosa *et al.*, 2006;

Kiritsakis *et al.*, 2002). The PV and FFA of the three different oils did not show significant differences among different treatments, whereas the whole fruit oil showed higher K_{232} and K_{270} values compared to the flesh only oil, which indicated that more primary and secondary oxidation had occurred in whole fruit oil. K values can be considered as a quality parameter in quality assessments of avocado oil (Green & Wang, 2022b).

Due to the abundant presence of phenolic compounds and pigments in avocado skin and seed (Araújo *et al.*, 2018; Ashton *et al.*, 2006; Dabas *et al.*, 2013; Ejiofor *et al.*, 2018; Kosińska *et al.*, 2012; Wong *et al.*, 2011), the analysis of avocado composition primarily focuses on the fatty acid profile, phenolics and pigments.

The fatty acid profiles of all the oils extracted with and without skin and seed showed no significant differences, and the percentage of each fatty acid identified were in the range for individual fatty acids proposed by the current CODEX standard (Codex Alimentarius Commission, 2021). The predominant fatty acid present was oleic acid. A second C18:1 isomer was also identified, reported to be cis-vaccenic acid, this was reported by Green and Wang (2022a).

Cárdenas-Castro *et al.* (2023) and Kosińska *et al.* (2012) have reported phenolics in extracts from skin and/or seed. Therefore, the total phenolic content was measured in the oils and a preliminary identification of individual phenolics present was carried out on each different oil. The total phenolics of the oil at time zero indicated that the flesh only oil had the lowest phenolic content (early season 12.03 mg/kg oil, late season 7.92 mg/kg oil, caffeic acid equivalent), followed by the flesh+skin oil (early season 20.70 mg/kg oil, late season 18.25 mg/kg oil, caffeic acid equivalent), with the whole fruit oil having the highest (early season 31.70 mg/kg oil, late season 25.94 mg/kg oil, caffeic acid equivalent). Shi *et al.* (2021) also measured the total phenolic content of avocado seed using the Folin-Ciocalteu method (64.55 mg GAEs/g), which was much higher than skin and pulp, which may explain the highest concentration of total phenolic content in whole fruit oil. In a typical olive oil, the mean total phenolics content was 483 mg/kg oil (Diamantakos *et al.*, 2021), therefore in comparison these values show low concentrations of phenolics. Compared to the late season flesh only oil, the early season oil showed significantly higher total phenolic concentrations, by 50% higher (p

< 0.05). The skin and seed are reported to have 25.3 and 9.5 mg/g phenolic in 'Hass' avocado, with some being oil soluble (Kosińska *et al.*, 2012).

In the whole fruit oil, the complexity of the individual phenolics and the quantity of phenolics significantly increased ($p < 0.05$), for instance the late season whole fruit oil contained 2.44 ± 0.60 mg kg^{-1} benzoic acid, which was significantly higher than its content in flesh only oil (0.07 mg kg^{-1}) ($p < 0.05$). Various phenolic compounds were found in flesh only oil, including p-coumaric acid, benzoic acid, ferulic acid, sinapic acid, rutin, cinnamic acid and quercetin, while after adding skin and seed, gentistic acid, vanillic acid, caffeic acid, epicatechin and kaempferol were also identified in the oils. Previous studies have indicated that avocado skin and seed contain caffeic acid, ferulic acid, vanillic acid, rutin, kaempferol and epicatechin (Jimenez *et al.*, 2021; Kosińska *et al.*, 2012; Pahua-Ramos *et al.*, 2012; Terasawa *et al.*, 2006; Tremocoldi *et al.*, 2018). Therefore, oil soluble phenolics in the avocado skin and seed were dissolved into the oil during the extraction process. Furthermore, the early season oil had a higher phenolic concentration compared to the late season oil for all three treatments.

Ashton *et al.* (2006) and Wong *et al.* (2011) reported on the pigments extracted into the oil with different levels of skin added to the malaxer. It was found that incorporating skin led to a slight elevation in the total chlorophyll content compared to oil from flesh only, which agreed with their results. However, a significant increase in total chlorophyll was shown when oil was extracted from whole fruit ($p < 0.05$). As for carotenoids in the oil extracted from flesh only, the concentration of lutein and β-carotene was significantly less than in the whole fruit ($p < 0.05$).

For the individual chlorophyll pigments, chlorophyll *a* (early season 20.68 mg kg^{-1}, late season 12.30 mg kg^{-1}), chlorophyll *b* (early season 19.53 mg kg^{-1}, late season 15.00 mg kg^{-1}), pheophytin *a* (early season 27.89 mg kg^{-1}, late season 32.65 mg kg^{-1}) and pheophytin *b* (early season 3.72 mg kg^{-1}, late season 3.88 mg kg^{-1}) in whole fruit oil have also shown a notable elevation compared to flesh only oil, consistent with the results of total chlorophyll. The inclusion of skin in the oil did not show significant differences ($p > 0.05$) in carotenoids and total chlorophyll content compared to flesh only oil, although there were higher concentrations of pheophytin *a* and lower concentrations of chlorophyll *a* in flesh and skin oil. This suggested that the addition of skin did not significantly affect the overall increase of pigments in avocado oil.

The variations in compositions among different oils indicate that while the addition of skin and seed does not have a pronounced impact on fatty acid profile, it increases the content of phenolics and pigments in the oil, which proves lipophilic substances in skin and seed can be extracted into oil during malaxing. Although the content of phenolic compounds significantly increased in whole fruit oil compared to flesh only oil, it remains considerably lower in comparison to extra virgin olive oil (Beltrán *et al.*, 2007; Siliani *et al.*, 2006). However, the carotenoid and chlorophyll content in whole fruit avocado oil is significantly higher than that in extra virgin olive oil (Mateos & García-Mesa, 2006).

The variation in chemical composition may have a significant impact on the quality and stability of avocado oil. PV, FFA and K values as the important quality parameters were measured during storage. FFA levels in all samples showed no significant changes during storage ($p > 0.05$), indicating the absence of lipid hydrolysis throughout the storage period. PV and K232 reflect the primary oxidation level of the oil, while K270 indicates the secondary oxidation level (Angerosa *et al.*, 2006; Elez-Martinez *et al.*, 2005; Kiritsakis *et al.*, 2002). At storage temperatures of 20°C and 30°C, a similar trend of increasing PV values was observed, with no significant differences between the different treatments ($p > 0.05$). However, after storage at 40°C, the PV and K values for the three oils increased at a much faster rate than at 30°C. The stored oil from whole fruit oil oxidised the fastest and reach a PV of 26.9 meq/kg after 111 days. The oil from flesh and skin was found to oxidise faster than flesh only oil but not as fast as whole fruit oil. The addition of skin had a small impact on the oil oxidation rate, whereas the inclusion of the seed accelerated the occurrence of oxidation reactions. Chlorophylls, pheophytins and lutein were considered to be prooxidant compound in edible oil (Subagio & Morita, 2003; Usuki *et al.*, 1984). Some studies have suggested that phenolics can also exhibit pro-oxidative behaviour under certain conditions (Decker, 1997). The addition of skin and seed provided higher content of phenolics and pigments, which could explain why the whole fruit oil has shown faster oxidation rate than the other two oils.

The greenness (a^*) of oils gradually decreased during storage, mirroring the changes in total chlorophyll content. This was a positive correlation between the green colour of the oil and its total chlorophyll content. Chlorophylls have been found to exhibit prooxidant activities in the oil (Choe *et al.*, 2019; Giuliani *et al.*, 2011), potentially leading to a faster rate of oxidation with higher chlorophyll content. Meanwhile, Park *et al.* (2013) and Zeb and Murkovic (2013) also reported the prooxidative properties of β-carotene in chlorophyll and oil. This is further supported by the previously mentioned higher PV and K values in whole fruit oil. Therefore, it can be seen that the highest oxidation rate in whole fruit oil was attributed to the interactive effects of its elevated phenolics and pigments content. Furthermore, in the study of the mass fraction of pyropheophytins, it was observed that the increase in pyropheophytins of different oils did not vary with the different oils at the same storage temperature. It can be concluded that the breakdown of chlorophyll is greatly influenced by temperature, with accelerated storage temperatures leading to faster decomposition rates. Therefore, avocado oil with higher concentrations of chlorophyll pigments, which can act as prooxidants and accelerate oxidation if conditions are favourable (warmer storage temperatures, oxygen, light etc) will lead to less stability during storage and short shelf life.

Accelerated oxidation reaction experiments using a Rancimat were conducted to assess the oil stability. The addition of skin and seed significantly increased the induction time of the oils, which indicated a higher antioxidant strength and greater stability. This was contradictory to the results found after the storage trials, which showed faster oxidation at 40°C. Hence the extraction of more phenolics into the oil and of pigments can result in oil that appears stable with a Rancimat but on storage they were found to oxidise faster.

Bitterness in edible oils is commonly associated with the content of phenolic compounds (Favati *et al.*, 2013; Siliani *et al.*, 2006). However, the total phenolic content in all three oils decreased with increasing storage time, but bitterness increased. Breakdown of phenolics into smaller structures could lead to an increase of absorbance of 225nm, corresponding to an increase in bitterness and also a decrease in total phenolics, which was observed (Section 4.3.1). The reason for this outcome remains unclear and further examination through sensory tests during storage is also required for bitterness.

Chapter 7: Conclusions and Recommendations

7.1 Conclusions

It was found in this study:

- Adding skin and seed during malaxing reduces oil yield per malaxer due to the lower quantity of flesh mass.
- The fat-soluble compounds in avocado skin and seed can be extracted into the oil during the malaxing process.
- Fatty acid profile of avocado oil was not affected by the addition of skin and seed.
- During oil storage, PV and K values increased but total phenolics and total chlorophyll declined over time, particularly at high temperatures, indicating higher temperature accelerated oxidation. Storing oil at 20°C or less is recommended, some decreased quality is observed at 30°C, and temperatures of 40°C are definitely detrimental to oil quality.
- Oxidative stability by Rancimat suggested that there would be an improvement in stability with added skin and seed, though these results contradicted the storage trial results.
- FFA showed no significant changes throughout the storage process, indicating the absence of lipid hydrolysis.
- The a^* (greenness) of avocado oil decreased during storage, aligning with the decline observed in total chlorophyll, indicating their positive correlation.

7.2 Recommendations

It is recommended to remove the skin and seed for extra virgin avocado oil production and store at 20°C or lower to ensure good oil stability.

The composition of avocado oils with and without skin and seed need to be further investigated to identify more of the phenolic compounds and pigments extracted into the oil.

Sensory analysis of avocado oils with and without skin and seed also needs to be evaluated to determine if including skin and seed during malaxing will affect the sensory profile of the oil. Stored oil made with skin and seed should also be evaluated as the extracted phenolics appear to degrade and polymerise and increase bitterness.

With good quality fruit, the oil extracted with skin and seed, can meet quality and CODEX standards (quality, fatty acid profile and sterol composition), more research is required to determine the impact of skin and seed on oil composition and quality.

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Appendix

Appendix I: Fatty acid profile raw data of early season and late season avocado oil under three treatments at time zero (mean ± SEM, n=3).

	Early season (%)	Late season (%)

	Flesh only	Flesh and skin	Whole fruit	Flesh only	Flesh and skin	Whole fruit
Palmitic acid (C16:0)	14.4 ± 0.1^{a}	17.3 ± 0.1^{c}	16.9 ± 0.1^{c}	14.4 ± 0.1^{a}	15.7 ± 0.1^{b}	15.5 ± 0.1^{b}
Palmitoleic acid (C16:1)	5.4 ± 0.1^{a}	6.8 ± 0.1^{b}	6.5 ± 0.1^{b}	5.4 ± 0.1^{a}	8.4 ± 0.1^{c}	8.5 ± 0.1^{c}
Stearic acid (C18:0)	0.4 ± 0.1a	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}
Oleic acid (C18:1)	61.3 ± 0.1^{b}	59.8 ± 0.2^{b}	59.6 ± 0.1^{b}	61.3 ± 0.2^{b}	53.1 ± 0.1^{a}	53.6 ± 0.1^{a}
Cis-vaccenic acid (C18:1)	6.3 ± 0.2^{a}	5.9 ± 0.1^{a}	6.3 ± 0.1^{a}	6.3 ± 0.1^{a}	7.9 ± 0.1^{b}	7.8 ± 0.1^{b}
Linoleic acid (C18:2)	11.6 ± 0.1^{b}	9.3 ± 0.1^{a}	9.8 ± 0.1^{a}	11.6 ± 0.1^{b}	14.1 ± 0.1^{c}	13.6 ± 0.1^{c}
Linolenic acid(C18:3)	0.6 ± 0.1^{a}	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}

Values significantly different in the row are noted with different lower case letters $^{a-c}$.