Converting Industrial Cold Pressed Avocado Oil Waste into Higher Value Products

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Thesis Abstract

Organic cold-pressed avocado oil (CPAO) has seen a steady incline in popularity over the past years due to its health promoting benefits and versatile culinary applications. However, the CPAO extraction process is relatively inefficient because of large amounts of by-product and organic wastes generated. To put this into perspective, for every 100 kg of avocado oil extracted; 570 kg of avocado wastewater (AWW), 350 kg of avocado skin and seeds and 190 kg of avocado pomace are generated. At present the pomace is utilised as animal feed but the skin, seed and wastewater are discarded into landfill. This disposal practice is of great concern as it causes pollution to the New Zealand environment and incurs high disposal costs to the avocado oil industry. A small body of studies have explored ways to utilise the avocado skin and seed. However, no research has been conducted on the use and application of AWW. Hence the aim of this study is to convert avocado skin, seed and wastewater into value added products. The success of this research would aid waste management in the avocado industry, as well as alleviate environmental impact from waste disposal and potentially create an avenue to generate additional income for the avocado oil industry.

Proximate analysis and antioxidant analysis of CPAO by-products were conducted. The largest by-product, AWW was successfully spray dried into powder using various temperatures ranging from 110˚C to 160˚C. The powders were then utilised as preservatives in pork sausages to prevent lipid oxidation.

While exploring for potential application of AWW powder, spray drying parameters were adjusted to increase yield of AWW powder with the addition of carriers and encapsulation techniques. AWW encapsulated with 5% whey protein isolate was then tested as a preservative in cooked pork fat. Findings showed that the effectiveness of AWW powder in preventing lipid oxidation was comparable to commercial preservatives.

Avocado seeds which account for approximately 13% of the by-products from industrial CPAO extraction was converted into an extruded snack using a friction cooker. Potential toxins such as amygdalin and persin were quantified in the seed snack. Both toxins were found in low concentrations would not have a negative impact on the human body if the snack was friction cooked and then consumed.

Overall, this thesis demonstrated the use of various modern technologies such as friction cooking, encapsulation, and spray drying to valorise the avocado by-products. Classical and current analytical techniques were also used in conjunction to the above technologies to provide an insight into various ways that CPAO by-products could be upcycled.

List of Figures

List of Tables

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to be the best of my knowledge and belief, 'Converting Industrial Cold Pressed Avocado Oil Waste into Higher Valued Products", has no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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Candidate contributions to co-authored papers

Chapter 3 - Published Permal, R., Leong Chang, W., Seale, B., Hamid, N., & Kam, R. (2020). Converting industrial organic waste from the cold-pressed avocado oil production line into a potential food preservative. Food Chemistry, 306, 125635. https://doi.org/10.1016/j.foodchem.2019.125635	Permal 90%, Kam. 4% Chang 3% Seal 1% Hamid 2%
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Table of Contents

1 Chapter 1: Introduction

Avocados have primarily been grown for the fresh fruit market which are subsequently sold for human consumption. Although there are 12 major cultivars of the avocado fruit, the main cultivars grown globally are the ‘Hass’ and ‘Feurte’, of which Hass is the most popular. Approximately 90% of avocado crops grown in New Zealand are Hass (Wong, Requejo-Jackman, & Woolf, 2010b). The popularity of Hass in New Zealand and worldwide is due to two reasons. Firstly, the cultivar has excellent yield potential and secondly, due to their thick skin, the fruit is less susceptible to postharvest, handling and transportation damage (Schaffer, Wolstenholme, & Whiley, 2013; Wong et al., 2010b). New Zealand farmers began to increase production of avocados from early 2000 which also led to the surplus of second-grade avocados that were not of export quality. Hence, an alternative use for second-grade avocados was to extract the nutrient dense oil inside the fruit. Avocado oil was not recognised as significant in the global market because the raw material cost was relatively high, and production was only in small scale. Therefore, producing a cold-pressed extra-virgin avocado oil, like extra virgin olive oil was employed to recover the value of the fruit.

The production and commercialisation of cold pressed avocado oil (CPAO) for the culinary market was facilitated by Olivado Ltd, a company based in Kerikeri, New Zealand (Eyres, Sherpa, & Hendriks, 2001). To retain the distinctive green colour of the oil as well as natural flavours aromas and nutritional profile, CPAO is removed from the flesh of Hass avocado using mechanical extraction methods rather than solvent extraction (Wong et al., 2008; Woolf et al., 2009) . Extraction of CPAO, is based on a continuous virgin olive oil extraction process which predominantly utilises mechanical means of extraction. The extraction of CPAO production includes washing, grinding, malaxing and centrifugation

(**Figure 1)**. During the malaxing stage temperatures are maintained at 45-50 ˚C for around 40 to 90 minutes (Eyres et al., 2001; Wong, Eyres, & Ravetti, 2014b).

CPAO has valuable nutritional properties, typical CPAO contains 76% monosaturated fats (oleic and palmitoleic acids), 12% polyunsaturated fats (linoleic and linolenic acids) and 12% saturated fats (palmitic and stearic acids). The oil is also high in pigments (chlorophylls and carotenoids), sterols and tocopherols (Eyres et al., 2001; Wong et al., 2010b). With the multitude of beneficial properties and a consumer perception towards natural products being organic and less 'polluted', production and market demand of CPAO has increased significantly over the past years (Gunstone, 2011).

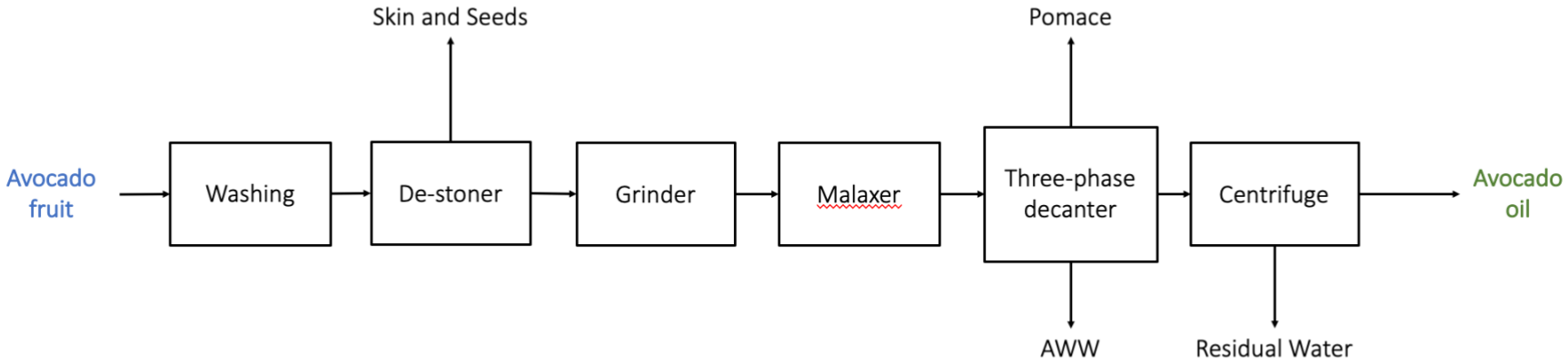


Figure 1. Flow chart of cold pressed avocado oil extraction. Waste streams include the skin, seeds, pomace, avocado wastewater (AWW), residual water. Adapted from Permal, Leong Chang, Seale, Hamid, and Kam (2020).

Ironically, extraction of CPAO leads to vast accumulation of organic waste, including the avocado seed, skin, pomace, and wastewater. These suboptimal counterparts of the avocado contain nutritious substances such as antioxidants, fats and vitamins, which are discarded directly into landfill (Domínguez et al., 2014). Several articles have looked at ways to upcycle these by-products, particularly avocado skin, and seed. Saavedra et al. (2017) proposed a method to dehydrate the avocado seed and skin using a convection oven and then grounding them into powder. The authors revealed that the dried powders were high in antioxidant activity and could be utilised as a storable commodity to enable their use as fortifying ingredients. Hatzakis, Mazzola, Shegog, Ziegler, and Lambert (2019) were successful in isolating perseaorangin, a natural orange pigment from the avocado seed, with potential applications as a dye. A small body of research also focused on the extraction of nutraceutical components from the avocado seed through fermentation and microwave assisted techniques (Araújo et al., 2020; Yepes-Betancur et al., 2021). With an abundance of articles on the skin and seed, not many have focused on how to utilise or treat avocado wastewater (AWW), the largest waste stream from CPAO production (Permal, Leong Chang, et al., 2020).

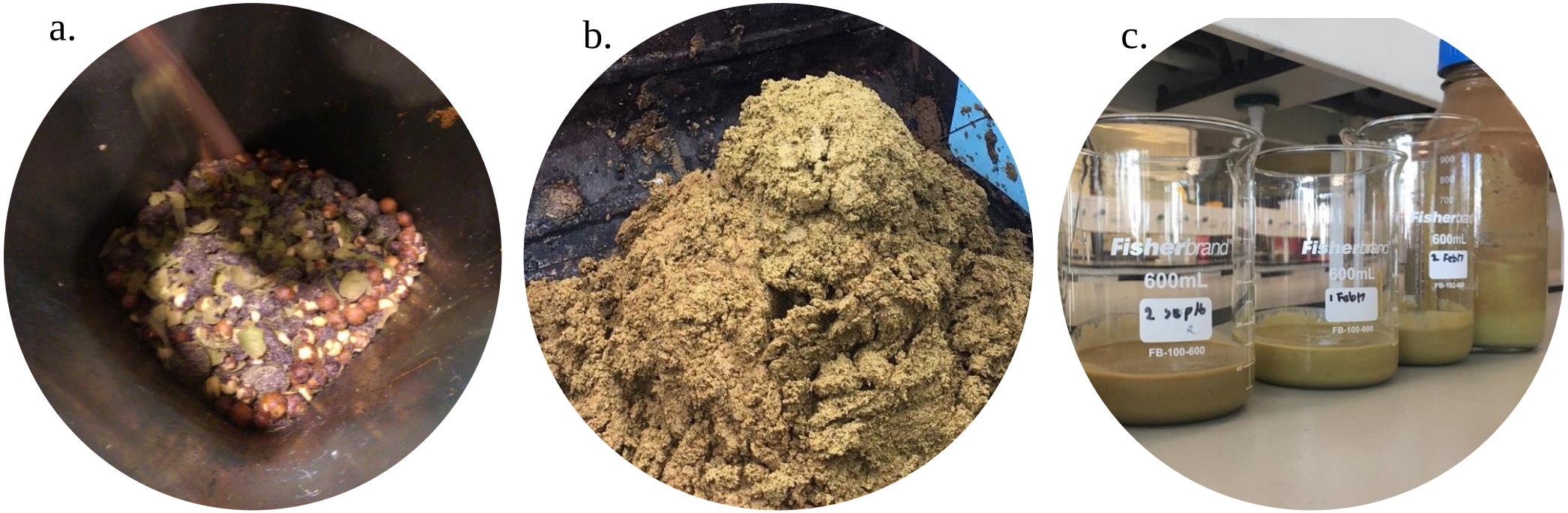


Figure 2. Avocado skin and seed (a), avocado pomace (b) and avocado wastewater (AWW) (c).

Chapter 1

A recent paper by Permal, Leong Chang, et al. (2020) conducted a mass balance of CPAO from Olivado Ltd's production line. The authors estimated that for every 1000 kg of fresh avocado, 78 kg of CPAO is produced. The remaining mass (922 kg) is accounted in the form of avocado seed, skin, pomace, and wastewater (**Figure 2**). At present, Olivado Ltd utilises the pomace as animal feed for livestock, however, avocado seeds, skin, and AWW are sent to the landfill. Disposing of these by-products, especially the AWW, comes at a cost to Olivado Ltd. The ultimate aim of this thesis is to explore ideas of valorising the aforementioned by-products into higher value commodity. The outline of this thesis will be presented in the following order:

Chapter 2: Literature Review

This chapter will serve as a literature review on the most current research in converting avocado by-products into something useful.

Chapter 3: Converting industrial organic waste from the cold-pressed avocado oil production line into a potential food preservative

This chapter was published in *Food Chemistry* and looked at the conversion of AWW into spray dried powder and utilising it as a food ingredient.

Chapter 4: Optimising the spray drying of avocado wastewater and use of the powder as a food preservative for preventing lipid peroxidation

This chapter was published to *MDPI Foods* and was an expansion of chapter 3. That presented the effectiveness of AWW and core chemical compounds (α-tocopherol) responsible for preventing lipids/oils from going rancid in foods. It also looks at optimisation of the AWW powder yield during the spray drying process.

Chapter 5: Converting avocado seeds into a ready to eat snack and analysing for persin and amygdalin

This chapter will explore the idea of converting avocado seeds into nutritious ready-to-eat (RTE) snacks. The chapter has been accepted for publication is *Food Chemistry*. Avocado seeds are common by-products that are often discarded into landfills. This study was the first to explore the potential of converting avocado seeds into a snack product using friction cooking. The study also looked at testing avocado seeds for amygdalin and persin, two naturally occurring toxins, in the avocado seed and avocado seed snack.

Chapter 6: Conclusion

Finally, this chapter will conclude the thesis by summarising all the findings from this PhD research.

2 Chapter 2: Literature Review

2.1 Brief history of the avocado fruit

The avocado (*Persea americana* Mill.) is from the Lauraceae family of the order Laurales, a pantropical family of about 2500 to 3000 species comprised mostly of trees and shrubs (Schaffer et al., 2013). According to Smith (1966), the earliest archaeological evidence of avocado use by humans was found from *Persea* cotyledons remains collected inside the Coxacatlan Cave in Tehuacan, Mexico, dated 7000 – 8000 BC. Smith (1966) explained that Coxcatlan caves are in arid areas, favouring food preservation. Interestingly, the avocados may not have been native to the immediate areas but rather gathered from more humid canyons from neighbouring mountains.

In the moist, lowland tropical zones around the Gulf of Mexico and the Pacific, numerous early civilisations thrived. This included the Mokaya, in the Soconusco region of Chiapas', and Guatemala's Pacific Coasts (1800 BC); the Olmec (1600 to 500 BC) in Tabasco and Gulf of Mexico Coast; and the Mayans in both of these areas, as well as in Guatemala, Honduras, El Salvador, Belize and Yutacan (Galindo-Tovar, Arzate-Fernández, Ogata-Aguilar, & Landero-Torres, 2007). In these areas, individuals would have come across *Persea* genotypes that were adapted to tropical, lowland areas. Schaffer et al. (2013) discussed, that as human population grew, life eventually become more sedentary, and human cultivation of *Persea* developed. There is also speculation that humans began to not only harvest but also cultivate and make selections from the genotypes encountered. Field work in Tehuacan by Smith (1966) showed an increase in cotyledon size of *Persea* over time, suggesting that some form of selection for larger fruits was occurring. The early emerging Meso-American cultures are recognised to have engaged in extensive trade from Central Mexico to Nicaragua and Pana

and are known to have obtained plants through these networks, originated as far south as the Amazon (Ogata, Gómez-pompa, & Taube, 2009). These trades are believed to have included exchange of the *Persea* germplasm (Galindo-Tovar, Ogata-Aguilar, & Arzate-Fernández, 2008). As a result of the trades, *Persea* was partially domesticated at least three times, due to geographically separation of the exchange. The domestication resulted in three recognised races of the avocado known as, the Mexican, the Guatemalan and the West Indian race or ecotypes of avocado (Cowan & Wolstenholme, 2016).

The first mention of the avocado was during the colonial era, by an English merchant named Hawkes, who was visiting Mexico in the mid-16th century. He referred to the fruit as 'alvacata', a misspelt term as Spanish was foreign to his English tongue (Popenoe, 1963). Subsequently, the fruit then appeared on several other reports in areas such as, Nicaragua, Jamaica and the Dominican Republic (Schaffer et al., 2013). The earliest verified introduction of avocado to California was in 1856 on the property of Dr Thomas J. White, who had obtained various tropical fruit species from Nicaragua. However, it is most likely that this introduction was unsuccessful as the trees were probably of West Indian descent (Condit, 1916). The first successful introduction of cold hardy avocados from Mexico is said to have been from Judge R.B. Ord of Santa Barbara in 1871; two of the three trees fruited and encouraged interest in avocado culture in southern California (Condit, 1916; Schaffer et al., 2013). As the 19th century came to a close, sporadic planting of seedling from particularly low-quality avocados existed in California. Then during the early 20th century with more seedling trees coming into bearing around southern California, the variability of quality was noticed, and the hunt for superior cultivars commenced. A small nursery industry was created in southern California where superior avocados were propagated by budding. One of these nurseries was West India Gardens. In efforts to find superior fruits to grow in California, the West India Gardens commissioned Carl B. Schmidt to travel and collect avocado budwood in

Mexico. Mr Schmidt sent back 41 selections, one of which was especially vigourous and given the name ‘Feurte’ which is Spanish for ‘strong’(Smith. N, Williams, Plucknett, & Talbot, 1992). This cultivar was released by West India Gardens in 1911 and by 1940 it occupied around 75% of the orchard area in California. The Feurte cultivar also gained success around the globe birthing new avocado industries in other sub-tropical Mediterranean and summer rainfall climates such as Israel, South Africa, Australia and Chile. While Feurte continues to be an important variety globally, it has been overtaken by another chance seedling that was created in southern California. The ‘Hass’ avocado is by far the most widely grown cultivar of *P.americana* in the world today. This cultivar was a Mexican-Guatemalan hybrid that originated around 1926 in La Habra Heights, California, when a graft had failed and the rootstock grew to fruiting (Schaffer et al., 2013). The owner of the tree, Rudolf Hass, realised that the fruit was superior to other cultivars that were available in the market, in terms of physical properties. He therefore, obtained a patent in 1935 (Smith. N et al., 1992). According to Schaffer et al. (2013) by 1990 ‘Hass’, had accounted for approximately 83% of the avocado production while ‘Feurte’ had dropped significantly to about 2%.

2.2 Introduction of avocados into New Zealand

When selected cultivars from California and Florida spread around the globe during mid to early 20th century, Commercial orchards were finally introduced to New Zealand (Schaffer et al., 2013). Literature by J. White (2001) stated that Len Grey of Gisborne was the first person to introduce avocados to New Zealand. Grey’s family orchard received some seeds distributed by the New Zealand Department of Agriculture in 1926, which started to produce fruit in 1935. Then in 1939 its fruits were marketed in New Zealand for the first time and with good consumer feedback, commercial production began the following year.

By the 1940's Len Grey imported some grafted trees from California and by the 1960's his orchard had grown to around 1000 trees. During the same period, Peter Kent, a nurseryman from Otumoetai, Tauranga, introduced Hass, Feurte and other varieties of avocados sourced from California in the 1950's. These trees were to become budwood for later plantation of avocados in the Bay of Plenty (J. White, 2001; Wratt & Smith, 2015).

The late 1970's and early 1980's experienced a boom of horticulture in New Zealand which was mostly centred towards the development of kiwifruit in the Bay of Plenty. However, small avocados plantings of mixed varieties were still grown. The increase in kiwifruit production resulted in investment in packhouses and infrastructure to handle this particular fruit. Fruit exporting businesses were established and were looking to take other types of crops on board. During this time there were a small number of commercial scale avocado orchards planted in the Bay of Plenty, Whangarei and Northland. As volumes grew, the New Zealand domestic market became lower priced, making export to Australia a viable option (J. White, 2001).

From the mid 1990's up until now, the New Zealand avocado industry has seen a strong consistent growth with increasing volumes and numbers of growers entering the industry adding additional new plantings. Gathering confidence from trading avocados with Australia, New Zealand began exporting avocados worldwide from 1999 and has continued to do so with increasing numbers of avocados each consecutive year (NZA, 2018; J. White, 2001).

2.3 Avocado varieties

There are three major botanical varieties or sub-species of the avocado fruit. The Mexican race has been referred to as *Persea americana var, drymifolia,* the West Indian race as *Persea americana var, americana* and the Guatemalan race as *Persea americana var, guatemalensis (Cowan & Wolstenholme, 2016).* **Table 1** shows that each of these various ecotypes and their

own characteristics. West Indian avocado varieties can be characterised as large, thick skin, low oil content (< 8%) and high water and sugar content. The Mexican variety have a high oil content (30%). The Guatemalan ecotype are recognised to have a 'nutty' flavour, desirable horticultural traits, and intermediate oil content (20%). Over the many years, several countries have participated in extensive selection and breeding programs that have produced fruit with improved qualities (Cowan & Wolstenholme, 2016; Schaffer et al., 2013). Yet, cultivars such as Pinkerton, Feurte, Ettinger and Hass, are amongst the most important commercial cultivars that resulted from chance seedlings. There are no known racial sterility barriers between the various avocado ecotypes, and as a result there has been a steady mixing of genes in areas where the different varieties grow together. Interestingly, the most important cultivars are hybrids having characteristics of more than one race. For example, the Feurte, is a Mexican and Guatemalan hybrid. Hass, long considered to be purely Guatemalan has now been shown to contain a small percentage of Mexican in the germplasm (Wood, 1984).

Table 1. Comparison of the three avocado races (Bergh & Ellstrand, 1986).

Trait	Mexican	Guatemalan	West Indian
TREE			
Climatic adaption	semitropical	subtropical	tropical
Cold tolerance	most	intermediate	least
Salt tolerance	least	intermediate	most
Hairiness	most	less	less
Leaf anise	present	absent	absent

Leaf colour	medium	often redder	paler
FRUIT			
Months to mature	6	12 or more	5
Size	small	variable	variable
Pedicel (stem)	slender	thick	nail-head
Skin thickness	very thin	thick	medium
Skin surface	waxy bloom	rough	shiny
Seed size	Large	small	variable
Seed cavity	loose	tight	variable
Seed surface	smooth	smooth	rough
Oil content	highest	high	low
Pulp flavour	spicy	often nutty	mild

2.4 Avocado growing season in New Zealand

The primary growing region of avocados in New Zealand is the Bay of Plenty and Northland (J. White, 2001). Hass avocados mature for around 9-18 months on the tree itself, increasing in size and oil content. Avocado fruits are unique as they only begin to ripen after they have been harvested. Unharvested fruits can remain on the tree even when the following year's fruits are developing and can remain on the tree for more than 18 months after flowering. Once harvested, the ripening process involves the softening of the flesh due to endogenous pectolytic enzyme activity (Wong et al., 2010b). In New Zealand, matured Hass avocados are harvested from September to April. Cowan and Wolstenholme (2016) explain that during this period the management of orchard are essential for quality fruits. Physiological factors such as temperature, irradiance, salinity, and water stress must be adjusted to produce commercially viable avocado fruits.

Hence, to grasp the growth cycle of an avocado orchard, Wolstenholme and Whiley (1989) carried out a study in order to construct a phenological model shown in **Figure 3.** The

point of interest in maintaining a healthy avocado tree is depicted by the two root growth periods in the spring and summer time as well as the alternating root flushes in early summer and autumn. Superimposed in **Figure 3** are reproductive events the fruit sets and flowers. These occur in the late winter and spring where two important periods of fruit drop occur (mid-summer and spring)

Figure 3. Phenology model developed for 'Feurte' Avocado trees. Growth in warm, subtropical climate in south-east Queensland, Australia (Schaffer et al., 2013)

Consequently, yield is regular from year to year in the absence of extreme weather conditions. However, the phenology of the avocado can vary depending on the environment. For example, in more stressful high altitude, semi-arid Mediterranean climates such as Israel, Spain, Chile and California, there are three root flushes, with the extra root flush during autumn. Flushes aid in flowering/fruiting sites for the following seasons and in doing so, maintain a good vegetative reproductive balance (Schaffer et al., 2013; Wolstenholme & Whiley, 1989). Additionally, Avocados trees, especially the 'Hass' cultivar is prone to alternate bearing or biennial cycles of a light, low yield 'off' crop year, followed by a heavy

or high yield ‘on’ crop year. Although in general, the on/off cycles are biennial, there are some cases an ‘on’ year can be followed by two or more consecutive ‘off’ years. Additionally yield reductions from the ‘on’ to ‘off’ year can be 100%, with no fruit in the year following the ‘on’ crop year. Alternate bearing in avocados are initiated by climatic events e.g. high or low temperatures, lack of rain during fruit set or bloom, resulting in low flower numbers or excessive flower and/or fruit abscission (Schaffer et al., 2013).

2.5 Avocado consumption and nutritional composition

During the past 20 years, avocado consumption has increased substantially, throughout the developed and developing world. The success for increased avocado production and consumption is due to market demands for natural and nutrient dense foods, coupled with research showing the immense health benefits of consuming avocados (Gunstone, 2011; W. Wang, Bostic, & Gu, 2010). Although avocados are primarily consumed fresh, substantial gains in the use of ‘value-added’ avocado products, for example, guacamole and avocado oil for culinary and cosmetic purposes suggest further market growth. (Schaffer et al., 2013).

Avocado consumers typically consume more nutrients such as potassium, dietary fibre, magnesium, and vitamin K and E compared to non-avocado consumers. The United States Nutrition Labelling and Education Act (NLEA) define the serving size of an avocado as 50 g or one-third of a medium fruit. A serving size of 50 g is defined by the FDA (2022) based on a 2,000 calorie diet. The FDA’s guideline proposes 40, 100 and 400 calories to be low, medium, and high, respectively. Placing one third of a medium avocado (50 g) at around a ‘low to moderate’ food serving regarding calories. However the National Health and Nutrition Examination Survey (NHANES), found that the average person consumes about one half of an avocado, about 66-75 g per day (Dreher & Davenport, 2013). Consuming one half (68g) of an avocado provides a considerably large dose of nutrients. According to **Table**

2, half of an avocado would provide about 24% of your recommended daily intake (RDI) of total fat, 30% RDI of vitamin B6 and 41% RDI of folate (MOH, 2022). Breaking down the fat content, Avocado contains 71% monounsaturated fatty acids (MUFA), 13% polyunsaturated fatty acids (PUFA) and 16% saturated fatty acids (SFA). As the avocado fruit ripens, the saturated fatty acids decrease and the monounsaturated oleic acid increases (Dreher & Davenport, 2013; Lu et al., 2009)

The significant levels of vitamin C can be found in avocado flesh, approximately 2.6 mg and 6.0 mg vitamin C per 30 g and one-half fruit respectively (**Table 2**). Studies by Berliner and Heinecke (1996) suggest that oxidative modification of low-density lipoproteins (LDL) and some other lipoproteins promote atherosclerotic vascular disease, a leading cause of death among persons with a Western lifestyle. Interestingly, evidence suggests that vitamin C may contribute to a vascular health and arterial plaque stabilisation. More specifically, in vitro studies have found that vitamin C at concentrations greater than 40 µmol/L inhibits the oxidation of LDL induced via free radical initiators, transition metals and activated human neutrophils and macrophages (IOM, 2000).

Vitamin C has been found to work in conjunction with vitamin E (α-tocopherol) employing a crucial role in recycling vitamin E to maintain circulatory antioxidant protection, potentially slowing the rate of LDL cholesterol oxidation the (IOM, 2000). Fortunately, avocados are one of the few fruits that contain significant levels of both these vitamins. **Table 2** estimates vitamin E values to be around 0.59 mg and 1.34 mg vitamin E per 30 g and one half avocado, respectively (USDA, 2019). Both Vitamin E and C have been found to retard the progression of the common carotid atherosclerosis, especially in men. However, clinical data over a period of 6 years, suggested that the combination of both these vitamins were not as statistically significant in women to show any plausible effect (Salonen et al., 2003).

According to X. Wu et al. (2004) avocados had the highest lipophilic total antioxidant capacity amongst commonly consumed American fruits ($ORAC_{FL}$ value of 5.52 µmol of Trolox equivalent (TE)/g of fresh weight (FW)), compared to tangerine, which had the lowest ($ORAC_{FL}$ value of 0.07 µmol of TE/g of FW). X. Wu et al. (2004) explains that the high lipophilic content could be the combined contribution of linoleic acid, vitamin E and carotenoids. Furthermore, research by Lu et al. (2005) emphasises that avocados are an important dietary source of xanthophyll carotenoids which has shown to inhibit the growth of prostate cancer cells when coupled in a diet of fruits and vegetables. Hass avocado carotenoid levels show an increase as the harvest season progresses from January to September in the northern hemisphere. An example of this variation, from the San Luis Obispo orchard in California, found the total carotenoid content of Californian Hass increase from 5.9 to 42.2 µg/g from early to late season (Lu et al., 2009).

Table 2. Chemical composition of Hass avocado flesh (California) flesh (USDA, 2019).

Nutrient/Phytochemical	Unit	Value Per 100 g	1 fruit 136.0 g	½ fruit 68 g	1 Serving (NLEA[1] serving) 50.0 g
Proximate					
Water	g	72.33	98.37	49.2	36.2
Energy	kcal	167	227	113.5	83.5
Protein	g	1.96	2.67	1.3	0.98
Total lipid (fat)	g	15.41	20.96	10.5	7.7
Carbohydrate, by difference	g	8.64	11.75	5.9	4.32
Fibre, total dietary	g	6.8	9.2	4.6	3.4
Sugars, total	g	0.3	0.41	0.2	0.15
Minerals					
Calcium, Ca	mg	13	18	9.0	6.5
Iron, Fe	mg	0.61	0.83	0.4	0.305
Magnesium, Mg	mg	29	39	19.5	14
Phosphorus, P	mg	54	73	36.5	27
Potassium, K	mg	507	690	345.0	254
Sodium, Na	mg	8	11	5.5	4
Zinc, Zn	mg	0.68	0.92	0.5	0.34
Vitamins					
Vitamin C, total ascorbic acid	mg	8.8	12	6.0	4.4
Thiamine	mg	0.075	0.102	0.1	0.037
Riboflavin	mg	0.143	0.194	0.1	0.071
Niacin	mg	1.912	2.6	1.3	0.955

Vitamin B-6	mg	0.287	0.39	0.2	0.143
Folate, DFE	µg	89	121	60.5	44.5
Vitamin B-12	µg	0	0	0.0	0
Vitamin A, RAE	µg	7	10	5.0	3.5
Vitamin A, IU	IU	147	200	100.0	73.5
Vitamin E (alpha-tocopherol)	mg	1.97	2.68	1.3	0.985
Vitamin D (D2 + D3)	µg	0	0	0.0	0
Vitamin D	IU	0	0	0.0	0
Vitamin K (phylloquinone)	µg	21	28.6	14.3	10.5
Lipids					
Fatty acids, total saturated	g	2.126	2.891	1.4	1.06
Fatty acids, total monounsaturated	g	9.799	13.327	6.7	4.9
Fatty acids, total polyunsaturated	g	1.816	2.47	1.2	0.91
Fatty acids, total trans	g	0	0	0.0	0
Cholesterol	mg	0	0	0	0

International units (IU) a unit commonly used in measurement of medications, vaccines, and vitamins. Retinol activity equivalents (RAE) a unit of measurement for vitamin A. Nutrition Labelling and Education Act (NLEA).

2.6 Avocado production and sales

Avocados are primarily grown as fresh fruit for consumption. According to the FAO (2018), Mexico is the largest supplier of avocados, producing 2,184,663 tonnes of avocado in 2018, while New Zealand only produced 22,608 tonnes (NZA, 2018). The FAO (2018) estimated that total world production for avocados in 2018 was approximately 6,407,171 metric tonnes, a 6.7% increase from the total production in 2017. Along with this growth there is a large supply of rejected fruits. Quality checks for reject grade avocado fruits are only based on cosmetic appeal such as the degree of skin defects, spotting, external rots, vascular browning, skin colour, and tissue break down (Schaffer et al., 2013). Production levels of avocado in New Zealand vary year to year depending on the season (some trees bear fruit biennially), weather and export markets (Wong et al., 2014b). According to the NZA (2021) New Zealand produced a total of 45,304 tonnes of avocado in 2020 to 2021. Almost an increase of 50% compared to production in 2017 to 2018. Of the total, 27,725 tonnes were exported and 17,578 were sold in the local New Zealand market.

With the large increase in avocado production there is a large increase in fruit production that do not meet export quality criteria, hence they are classed into three groups. Class 1 avocados are exported, class 2 are sold in New Zealand and class 3 are sold for processing. Unfortunately, the relatively small population of New Zealand can only consume a limited amount of these reject grade avocados. If not utilised, the excess of class 2 and 3 of avocados would likely to result in a drop in avocado prices making the fruit less economical for growers (Eyres et al., 2001). Since there is a growing demand for avocados for culinary applications such as guacamole products and avocado oil. The ‘Hass’ cultivar makes up over 90% of the avocado industry and contains as much as 32% oil (Wong et al., 2014b). Therefore, extracting oil from the reject grade avocados would only add value to the avocado

industry. Eyres et al. (2001) reported that only after one season, avocado growers had seen a positive effect of extracting oil as an alternative to supplying the domestic market with low-grade fruit. The 2000 and 2001 seasons saw approximately 1200 tonnes of reject grade avocado fruits processed, yielding around 160 tonnes of oil. This conversion of low-grade avocado to oil was estimated to benefit the New Zealand avocado industry by approximately US$500,000 between 2000 and 2001.

2.7 Cold-pressed avocado oil (CPAO)

2.7.1 CPAO background

In the past, avocado oil had been extracted using only chemical solvent (e.g., hexane and petroleum ether) methods at varied temperatures. The extracted oil would be refined, bleached, and deodorised, leaving a yellow to almost colourless oil, since dark green pigments were removed during bleaching. These refined avocado oils were predominantly used for cosmetic purposes rather than for consumption.

Cold pressed avocado oil (CPAO) on the other hand, is a method of avocado oil extraction that has been around for more than 30 years and contrary to refined avocado oil, contains a higher concentration of pigments while maintaining its emerald green hue, as shown in **Figure 4** (Wong et al., 2008). Centrifugal force separation of avocado oil was introduced in the late 1980's by Werman and Neeman (1987) in Israel. They found the most efficient extraction temperature to be 75˚C with an oil yield of 64.5%.

Figure 4. Extra virgin avocado oil or CPAO and Refined avocado oil (Pradhan, 2022).

Following improvements on the extraction process of this type of oil was later carried out and facilitated by Olivado Ltd (Northland) (Eyres et al., 2001). Read (2002) reported that Olivado Ltd was originally established in 1998 with the aim of setting up a commercial olive grove and olive oil processing plant at Kerikeri. However, research suggested using avocados to produce an oil similar to olive oil during the olive off season would be profitable for the company. Taking this into consideration, Olivado Ltd launched extra-virgin avocado oil in November 2000, to be sold as a culinary oil for cooking and salad dressing. This project was developed in conjunction with Alfa Laval, a leading food processing company. Alfa Laval utilised its significant experience and technological experience in cold pressing extra-virgin olive oil (EVOO) to assist in developing a novel extraction method to obtain high quality, edible avocado oil. Commercial production of CPAO was first launched by Olivado Ltd

(Northland) and then The Grove NZ Avocado Oil (Bay of Plenty). CPAO, like EVOO, is not refined and maintains the chemical, organoleptic flavour profile of the fresh avocado fruit (Costagli & Betti, 2015; Wong et al., 2008).

2.7.2 CPAO extraction process

Lipids in avocados are stored in two cell types, parenchyma cells and idioblasts both in the mesocarp. The parenchyma cells within the mesocarp contain large lipid droplets (**Figure 5)** in the cytoplasm, primarily comprised of triglycerides (Qin & Zhong, 2016). As the avocados ripen, there is a rise in cell enzyme activity causing its pectin cells to de-esterify and de-polymerise. This causes parenchyma cell walls to soften, rupture and release lipids (Wong et al., 2008)

Idioblasts are a cluster of cell walls bound by protopectin in the middle lamella. The cells contain one large oil sac surrounded by a thicker cell wall than that of parenchyma cells (**Figure 6**). Histochemical tests have indicated that the idioblast cells contain alkaloids, sesquiterpene hydroperoxides and other terpenes (Platt & Thomson, 1992). Qin and Zhong (2016) expressed that these specialised cells proved difficult to rupture during CPAO oil extraction due to the thicker cell walls and immunity to activity of some enzymes. The authors suggested that increasing oil release from cellular bodies of the avocado flesh and idioblast cells would require, mechanical, enzymatic and thermal pre-treatments in combination to rupture the cell structures.

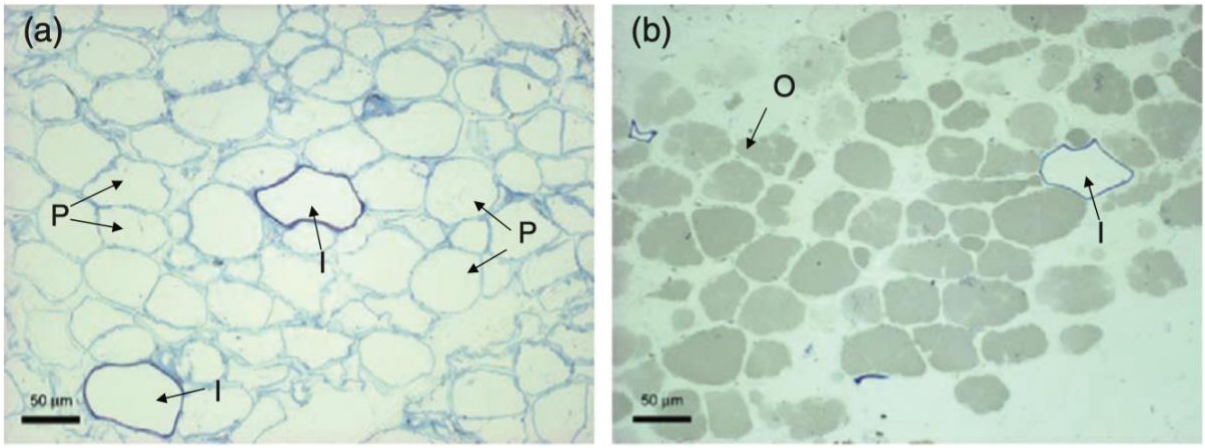


Figure 5. Microstructure of avocado flesh (a, b). Image (a) is samples embedded in London Resin (LR) White resin; cell walls stained blue in LR white embedded tissue. Image (b) is from samples embedded in Spurr's resin. Lipid in parenchyma cells show grey coloration in Spurr's embedded material. I, idioblast cells; P, parenchyma cells; O, oil droplets from parenchyma cells (Yang et al., 2018).

According to Wong et al. (2008) the seed is usually removed before processing, however, around 10% of avocado skin may be added to the malaxing process. The concentration of pigments extracted into oil is influenced by the amount of skin included at the malaxing step, as concentration of chlorophylls and carotenoids are highest in the skin (Wong et al., 2008). The seed and peel of the avocado fruit only contain 1% and 2.9% fat respectively (Saavedra et al., 2017). Therefore, most commercial avocado oil is extracted from the flesh. Qin and Zhong (2016) explain that fat availability in avocado flesh is dependent on the cultivar and timing of harvesting. For example, lipid content in Hass and Feurte cultivars during the early season typically range from 17.1 and 17.6% to 32.0 and 22.8% respectively in the late season (Slater, Shankman, Shepherd, & Alfin-Slater, 1975; Wong et al., 2014b).

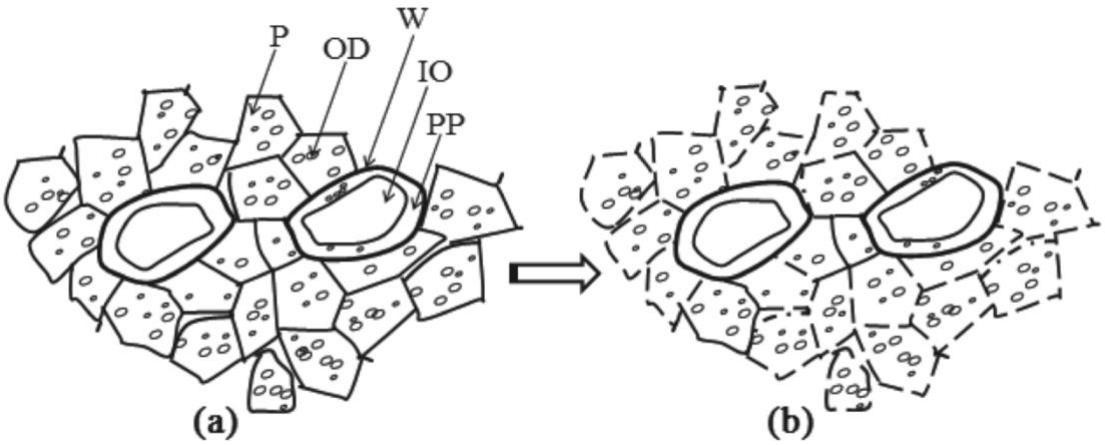


Figure 6. Mesocarp cells during ripening. a – unripen mesocarp cells; b – ripen mesocarp cells; P – parenchyma cell; W, idioblast wall, OD – oil droplet; PP – protoplasm; IO - idioblast oil sac (Qin & Zhong, 2016).

Extraction of CPAO is carried out using a similar process to olive oil. The Hass variety is most utilised in commercial cold pressed extractions, due to its high lipid content. **Figure 7** shows how this process occurs. First, the whole fruit washed thoroughly in a two-stage washing system. Then the fruit is destoned by separating seed and 90% of avocado skin from the pulp. To extract avocado oil, the pulp and skin then follow through a disk crusher, where it is simultaneously sliced and crushed, rupturing the cell walls, and releasing oil droplets in the parenchyma cells. Subsequently a crucial stage of CPAO known as malaxing then takes place. At this stage the avocado paste is continuously stirred at a controlled temperature ranging between 40-50˚C. Malaxing aids in agglomerating the oils ruptured from the parenchyma cell in the pulp emulsion. Next, the oil is separated from solid and liquid phases in a decanter centrifuge. Usually, a further 10 to 20 % of water is added (adjusted to the same temperature as the malaxing mash), aiding in separation of different liquid densities. Majority of the solids are separated at the decanter, however, the CPAO leaving out of the decanter contains residue water and solids that need to be further polished by vertical centrifugation (Costagli & Betti, 2015).

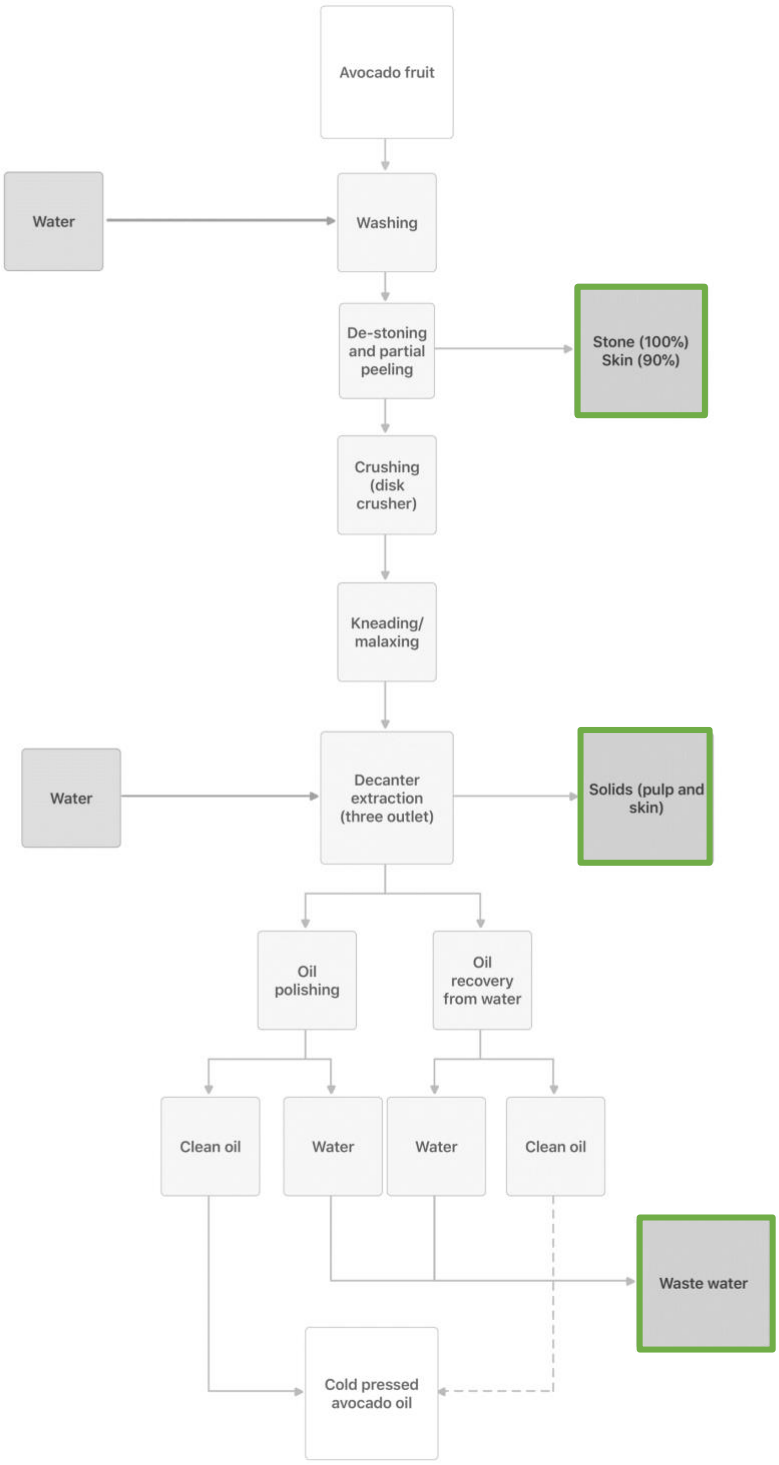


Figure 7. Flow chart of cold pressed avocado oil extraction. By-products include the seed/stone, solids – pulp, skin, and wastewater. Boxes outlined in green represent the by-products of CPAO (Costagli & Betti, 2015).

The pigments that predominantly influence the colour of CPAO is chlorophylls. Chlorophyll pigments are especially reactive under light and are known to degrade when exposed to oxygen (Wong et al., 2008). Therefore, to minimise this damage occurring to the oil post extraction, Olivado incorporates a dual nitrogen sparging system. Essentially, after the oil has been processed, it is sent into a primary storage tank where nitrogen is flushed into the oil. Sparging removes any dissolved gasses, most importantly oxygen, and replaces it with nitrogen, an inert gas. The use of nitrogen delays rancidity of the oil and stabilises the concentration of chlorophylls (Wong et al., 2014b). The oils are then sent to a secondary tank and again sparged with nitrogen. The CPAO is then packaged into dark bottles to prevent lipid peroxidation from light.

2.8 CPAO by-products

The production and processing of CPAO, leads to an abundance of organic waste that is sent directly into landfill. To put this into perspective Permal, Leong Chang, et al. (2020) carried out a flow diagram to detail the exact amount of waste that is produced from the Olivado's CPAO processing factory. The authors reported that for every 1000 kg of fresh avocado, only 78 kg of CPAO is produced. The remaining mass is accounted for by-products of CPAO in the form of seed (121 kg), skin (153 kg), pomace (150 kg), wastewater (448 kg) and residual water (50 kg). Many studies have been conducted to valorise these by-products, especially the avocado skin and seed. However, the largest by-product of CPAO (by volume) is avocado wastewater which, has not been gained much attention. The following sections will aim discuss the current research surrounding by-products of CPAO and how they may be utilised.

2.9 Valorising CPAO by-products

2.9.1 Pomace & wastewater

During CPAO production, the avocado pomace (exhausted pulp and residual skin; fibrous material) is expelled at the decentring stage along with avocado wastewater and accounts for approximately 16% of the total waste produced. Proximate analysis of the pomace shows that it is comprised of 72.1% dietary fibre 0.6% available carbohydrates, 6% protein, 6.4% protein, 6.4% ash and 9.3% lipid (Permal, Leong Chang, et al., 2020). Due to its nutritional content, avocado pomace can be used as animal feed mainly for bovine.

In recent work, Züge, Maieves, Silveira, Silva, and Scheer (2017) presented a strategy to utilise avocado pomace by extracting avocado phospholipids and incorporate it into food as an emulsifier. The author points out that avocado lipid fractions are rich in phospholipid, however, majority of these compounds are frequently discarded with the residual pulp during the oil extraction process. Extraction of phospholipids was facilitated through a mixture of methanol/chloroform and acetone followed by centrifugation and finally lyophilisation. The authors concluded that emulsions using 1 wt% phospholipids in commercial soy oil, presented an inversion interval (point at which an O/W emulsion converts to W/O) between 60 and 70 vol% oil phase. At this concentration, emulsions with 60 vol% oil phase had the highest stability. Therefore, phospholipids extracted from avocado pomace were capable of forming emulsion and maintaining stability for oil-in-water emulsions.

To the best of our knowledge, no research or studies have documented the use of avocado wastewater, the largest by-product of CPAO production. At present, Olivado Ltd seeks external services to remove the wastewater from their factory. The wastewater is very high in organic and lipid content and must be disposed of safely. Moreover, the residual wastewater that is produced from CPAO extraction can disposed of directly into the drains as it contains significantly lower organic content compared to wastewater.

2.9.2 Avocado skin

The skin of avocado fruits are rich in nutrients and contain a multitude of phytochemicals that may be efficiently used as drugs or as a food supplement (Kowalska, Czajkowska, Cichowska, & Lenart, 2017). Avocado peel has been found to contain high amounts of carotenoids, phenolic compounds and chlorophyll compared to the avocado pulp. The total carotenoids and chlorophyll content are shown in **Table 3**. From the data, it appears that chlorophyll in non-Hass avocado varieties is highly concentrated in the peels. Alternatively, Hass avocados were high in anthocyanin content, explaining its dark brown peel (W. Wang et al., 2010). A. F. Vinha, J. Moreira, and S. V. P. Barreira (2013) reported a total carotenoid content of 25.85 ± 1.17 µg β-carotene equivalents (BCE)/g FW in avocado skin, which was significantly different what was reported by Wong et al. (2010b) in **Table 3**. The difference in carotenoid concentration may be due to the growing environment of avocado orchards, which play a fundamental role in plant metabolism and evidently the chemical composition of the avocado (Schaffer et al., 2013). Hass carotenoid content reported by W. Wang et al. (2010) were from San Francisco and those reported by Ana Ferreira Vinha et al. (2013) were from Portugal. Interestingly, antioxidant capacity of avocado peels from different varieties have shown to be several folds higher than those reported for raw blueberries (5.3 GAE mg/g and 65 65 µmol TE/g ORAC), a fruit well known for its higher antioxidant capacity (W. Wang et al., 2010; X. Wu et al., 2004)

Table 3. Total pigment content (chlorophyll & carotenoid), total phenolic content (TPC), radical scavenging activity and oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), of various avocado portions from different cultivars. Note: Gallic acid equivalent (GAE), Trolox equivalent (TE)(Wong et al., 2010b).

Portion	Cultivar	Total Chlorophyll (µg/g)	Total carotenoid (µg/g)	Total phenolic content (mg GAE/g)	ORAC (µmol TE/g)	DPPH (µmol TE/g)
Seeds	Slimcado	0.2 ± 0.1	0.7 ± 0.1	19.2 ± 3.3	229.0 ± 16.2	128.3 ± 17.4
	Loretta	1.1 ± 0.4	1.2 ± 0.2	31.5 ± 2.2	299.0 ± 17.6	159.7 ± 18.5
	Hass	41.2 ± 5.7	6.3 ± 0.9	51.6 ± 1.6	428.8 ± 12.0	164.6 ± 5.1
Skin	Slimcado	34.8 ± 1.7	9.3 ± 1.1	4.6 ± 0.3	58.2 ± 1.3	39.7 ± 5.3
	Loretta	47.4 ± 2.9	17.3 ± 1.3	7.6 ± 0.8	92.6 ± 9.5	38.0 ± 2.3
	Hass	28.8 ± 6.2	15.2 ± 2.7	12.6 ± 0.3	631.4 ± 4.2	189.8 ± 10.8
	*Hass	-	25.85 ± 1.17	-	-	-
Pulp	Slimcado	3.7 ± 0.2	1.5 ± 0.2	1.0 ± 0.1	4.7 ± 0.2b	1.3 ± 0.2
	Loretta	2.7 ± 0.7	2.4 ± 0.4	1.0 ± 0.1	3.9 ± 0.5	0.4 ± 0.1
	Hass	28.7 ± 3.3	7.1 ± 0.6	4.9 ± 0.7	11.6 ± 0.4	1.3 ± 0.1

Data are mean ± standard deviation on fresh weight basis.
* Data from Ana Ferreira Vinha et al. (2013)

A study by Rotta et al. (2015) investigated avocado peel as tea and compared its antioxidant profile amongst other popular tea leaves (**Table 4**). Ferric reducing antioxidant power (FRAP), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and total polyphenol content (TPC) analysis in avocado peel tea stored for 45 days at room temperature showed no statistical difference in values from day 0 to day 45. The statistical analysis indicated that avocado peel tea was stable in tea sachets at room temperature. Interestingly, results of the DPPH assay indicated no statistical differences between mate tea and avocado peel tea. Mate tea is primarily consumed in South America for its antioxidant properties (Berté, Rodriguez–

Amaya, Hoffmann-Ribani, & Junior, 2014). Hence, a market for utilising avocado peel as tea could be a possibility.

Table 4. TPC, DPPH and FRAP assay of avocado peel teas (APT) and commercially sold teas (Rotta et al., 2015).

Tea Leaf	**TPC (mg GAE L^{-1})**	**FRAP (µmol $Fe_2SO_4.7H2O$ L^{-1})**	**DPPH (µmol TE L^{-1})**
APT 0 days	123.6 ± 4.6	2,166 ± 35.5	1954 ± 87.9
APT 45 Days	110.2 ± 2.6	2,166 ± 35.4	2518. ± 192.6
Mate tea	176.7 ± 6.1	3,477 ± 169.6	2858 ± 14.9
Apple tea	20.7 ± 0.9	2,777 ± 106.3	13497 ± 696.6
Green tea	493.8 ± 10.2	12,341 ± 344.2	2409± 86.1

Particularly, procyanidins have demonstrated antitumoral properties, anti-inflammatory effects and prevention of oxidative processes in key cellular structures (Cerda-Opazo, Gotteland, Oyarzun-Ampuero, & Garcia, 2021). The antioxidant effect of procyanidins from avocado peel have been reported by Ana Ferreira Vinha et al. (2013) and W. Wang et al. (2010). However, no studies have looked at the anticancer activity of avocado fruit peel directly. This may be attributed to the instability of procyanidins during application. Although, procyanidins have biological potential, their use in oral formulation is limited by structural characteristics. They have poor water solubility, rapid metabolism, and low bioavailability. In addition, the bitterness and astringency of the compounds limit its use as a food product. Looking to overcome this issue, a study by Cerda-Opazo et al. (2021) encapsulated a freeze dried procyanidin extract (Proc), from avocado peel into an oil-in-water (O/W) nano-emulsion (Proc-Nem). The Proc-Nem was stable under various biological and storage conditions and could be converted to a water-reconstitute powder. Proc-Nem was safe in non-cancerous cells (HEK293) and effective at killing cancerous cells (B16F10 melanoma

cells) by preventing their malignant migration. The authors explained that there are no studies explaining the mechanism of absorption of Proc in the B16F10. However, they had hypothesized that Proc-Nem may interfere with the redox-balance (generation and elimination of reactive oxygen/nitrogen species) of tumour cells, contributing to a decrease in cellular activity of B16F10 cells. Yet, the formula has yet to be tested in food matrices and *in vivo* experimentation in cancer models.

To reinforce the sustainability in extraction of polyphenols and phytochemicals from the avocado peel, Trujillo-Mayol, Céspedes-Acuña, Silva, and Alarcón-Enos (2019) applied concepts of green chemistry to reduce economic and ecological negative impact. The authors focused on utilising ultrasound-assisted extraction (UAE), microwave assisted extraction (MAE) and a combination of the two U-MAE. Due to low cost, easy scalability, and versatility to extract various molecules of different polarities, these methods offer advantages over pressurized liquid extraction, subcritical and supercritical fluids extraction, and accelerated solvent extraction (Nayak et al., 2015; Trujillo-Mayol et al., 2019). **Table 5** shows that the combination of 15 minute sonication followed by 95.1 s of microwaving gave the highest extraction yield (25.3%), total polyphenol content (TPC) (281.4 mg gallic acid equivalents (GAE)/g dry extract (DE)), total flavonoid content (TFC) (62 mg quercetin equivalents (QuE)/g DE), total anthocyanin content (TAC) (4.8 mg cyanidin3-O-glucoside/g DE) and 2,2-diphenyl−1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and lipid peroxidation (LPO) (779.1, 167 μg trolox equivalent anti-oxidant capacity (TEAC)/g DE and 70% respectively). Interestingly, all avocado peel extracts (APE) showed antimicrobial activity, specifically against foodborne bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella* Spp (Trujillo-Mayol, Casas-Forero, Pastene-Navarrete, Lima Silva, & Alarcón-Enos, 2020). Later, in efforts to reduce oxidation and formation of harmful compounds from cooking, Trujillo-Mayol et al. (2021)

incorporated APE (0.5% and 1%) into beef and soy burgers. The authors reported that addition of 0.5% APE inhibited the formation of heterocyclic aromatic amines (HAs) and acrylamide in both beef and soy burgers. Addition of APE affected the colour of both meats; however, it did not have an impact on consumer preference.

Table 5. Phytochemical and antioxidant attributes of avocado peel dry extracts (Trujillo-Mayol et al., 2019).

Extraction Method	Phytochemical attributes				Antioxidant Activity		
	Yield %	TPC (mg GAE/g DE)	TFC (mg QuE/g DE)	TAC (mg cyanidin-3-*O*-glucoside/g DE)	DPPH (µg TEAC/g DE)	FRAP (µg TEAC/g DE)	LPO (%)
UAE	16.6 ± 0.6a	270.4 ± 3.6a,b	54.1 ± 1.6a	2.3 ± 0.0b	772.2 ± 2.1a	161.7 ± 0.9a	59.17 ± 3.27a
MAE	16,0 ± 0.1a	274.9 ± 2.2a	54.1 ± 1.6a	4.4 ± 0.1a	775.8 ± 2.6a	157.8 ± 0.6a	61.05 ± 1.05a
U-MAE	25.3 ± 0.6b	281.4 ± 0.2c	62.0 ± 0.4b	4.8 ± 0.1c	779.1 ± 0.6a	167.0 ± 2.3b	70.03 ± 0.62b
Maceration	7.8 ± 0.1c	257.2 ± 7.5b	57.1 ± 0.9a	2.7 ± 0.1d	774.6 ± 0.7a	150.2 ± 0.8c	63.51 ± 1.05a

Note: Analysis was performed on bases of dry extract (DE). Average data are mean ± standard deviation (two replicates, n = 3 each). Letters within the same column indicate significant difference ($p < 0.05$) of means according to Tukey's test.
Abbreviations: MAE microwave assisted extraction; UAE, ultrasound assisted extraction; U-MAE, combined method; TPC, total polyphenol content; TFC, total flavonoid content; TAC, total anthocyanin content; DPPH, 2,2-diphenyl−1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; TEAC, trolox equivalent antioxidant capacity: GAE, gallic acid equivalents; QuE, quercetin equivalents; LPO, lipidic peroxidation inhibition.

There is a growing interest in preparing substitutes for activated carbon, a conventional adsorbent to remove dyes from colour effluents or wastewater. In the past, activated carbon was prepared from wood/coconut (45%), coal/lignite (42%) or animal bones and was designed as mineral carbona, vegetal carbona and bone char respectively (Elizalde-González, Mattusch, Peláez-Cid, & Wennrich, 2007). Although efficient, this practice was costly, therefore, various studies have looked at tackling industrial effluents using agro-industrial waste as an adsorbent (Contreras, Sepúlveda, & Palma, 2012; Elizalde-González & Hernández-Montoya, 2009; Elizalde-González et al., 2007). Turning to avocado waste, Palma, Lloret, Puen, Tobar, and Contreras (2016) utilised avocado peel as a precursor for carbonaceous solid to be used as an adsorbent. The research reported the most optimal carbonization conditions were found at 900˚C and 65 min, generating an adsorbent with a surface area of 87.52 $m^2 g^{-1}$ BET (Brunauer, Emmett and Teller method, for nitrogen adsorption isotherm analysis), a mesopore volume of 74% and zero-point charge at 8.6. The carbonaceous material was successful at removing naphthol blue black and reactive black 5 using 10 mg L^{-1} and 20 g L^{-1} of dye and solid, respectively. Basic Blue 41 on the other hand was eliminated using 13.4 g L^{-1} of avocado-based adsorbent.

2.9.3 Avocado seed

Like avocado peels, avocado seeds are a potential candidate for polyphenol extraction. A number of studies have reported its high antioxidant capacity as well as large amounts of extractable polyphenols (Segovia, Corral-Pérez, & Almajano, 2016). W. Wang et al. (2010) reported TPC in Hass peels were lower than the seeds (12.6 and 51.6 mg GAE/g, FW basis). Additionally, a high phenolic content of 88.2 mg GAE/g FW basis, in avocado seeds was also observed in an earlier study by Soong and Barlow (2004). W. Wang et al. (2010) summarised that the phenolic content in seeds, peels and pulp were approximately 64%, 23% and 13%

respectively, of the whole fruit. Whereby the seed alone contributed approximately 57% of the fruit's antioxidant capacity and the peel approximately 38%. To explore some of these phenolic compounds, Araujo et al. (2021) identified 20 phenolics and other polar compounds in avocado seed extracts with microwave assisted extraction (MAE) using ethanol by reverse phase-high performance liquid chromatography electrospray ionization mass spectrometry (RP-HPLC-ESI-MS). A summary of the phenolic compounds found in avocado seeds are summarised in **Table 6.** Although the authors did not provide the exact concentration, they did identify compounds that were present in high concentrations, specifically 12 procyanidins, timers and dimers in different isomer forms as well as organic acids such as 3-pcoumaroylquinic acid, 3-O-caffeoylquinic acid and caffeoylquinic acid, catechin, epicatechin, gallate and a rare alcoholic sugar, persitol. Thus, the high antioxidant activity of avocado seed extracts can mainly be associated with the presence of procyanidins and catechins which are compounds with a high free radical reduction capacity (Araujo et al., 2021).

Table 6. Identification of polar and phenolic compounds in extracts from avocado seeds with ethanol using RP-HPLC-ESI-MS (Araujo et al., 2021).

Peak	Proposed compound	RT (min)	m/z	Molecular formula
1	Perseitol	2.742	211	$C_7H_{16}O_7$
2	Proc. trimer B	12.174	864.8	$C_{45}H_{38}O_{18}$
3	Hydroxytyrosol glucoside	13.273	315	$C_{14}H_{20}O_8$
4	3-O-Caffeoylquinic acid	15.023	352.9	$C_{16}H_{18}O_9$
5	Proc. dimer B	16.98	576.9	$C_{30}H_{26}O_{12}$
6	3-p-Coumaroylquinic acid	17.891	336.9	$C_{16}H_{18}O_8$
7	(+)-Catechin	18.707	288.9	$C_{15}H_{14}O_6$
8	Proc. trimer B	19.291	864.9	$C_{45}H_{38}O_{18}$
9	Caffeoylquinic acid	19.658	352.9	$C_{16}H_{17}O_9$
10	Proc. trimer B	20.168	864.8	$C_{45}H_{38}O_{18}$
11	Proc. dimer B	20.605	576.9	$C_{30}H_{26}O_{12}$

12	Proc. dimer B	21.237	576.8	$C_{30}H_{26}O_{12}$
13	Proc. trimer B	21.987	864.7	$C_{45}H_{38}O_{18}$
14	(−)-Epicatechin	22.888	288.9	$C_{15}H_{14}O_{6}$
15	Proc. trimer A	23.271	862.7	$C_{45}H_{36}O_{18}$
16	Proc. trimer A	24.376	862.8	$C_{45}H_{36}O_{18}$
17	Proc. trimer A	26.249	862.7	$C_{45}H_{36}O_{18}$
18	Proc. trimer A	27.024	862.8	$C_{45}H_{36}O_{18}$
19	(Epi)catchin gallate	27.832	440.9	$C_{21}H_{30}O_{10}$
20	Proc. trimer A	29.188	862.7	$C_{45}H_{36}O_{18}$

With an abundance of phenolic content, Gómez, Sánchez, Gallego Iradi, Mohd Azman, and Almajano (2014) incorporated lyophilized polyphenol extract from dried avocado seeds. The authors measured its effect on delaying oxidation for oil-in-water emulsions in beef patties over 8 days. It was observed that there was no difference between the use of lyophilized extract at 0.1% or the commercial synthetic antioxidant, BHA (butylated hydroxyanisole). To characterise some of these lipophilic compounds, Soledad et al. (2021) detected 16 chemical compounds were determined using gas chromatography-mass spectrometry (GC-MS) shown in **Table 7** below. The chemical profiling indicated that avocado seed contained sesquiterpenoids, polyunsaturated fatty acids and unsaturated fatty acids esters. Moreover, the extracts also presented phenolic compounds with antimicrobial activity against *Salmonella enterica* subsp. enterica serovar Typhimurium and *Staphylococcus aureus*. Acetone extraction provided higher antioxidant capacity than ethanol (212.75 mg TE/100 g and 183 mg TE/100 g respectively), further signifying its usefulness in food products as a preservative.

Table 7. Chemical compounds, retention time of acetone and ethanol extracts of avocado seed (*Persea americana* cv. Criollo sp.) (Soledad et al., 2021).

Peak	RT	Component	% area acetone	% area ethanol
1	6.34[A]	Isoestragole	3.07	ND
2	6.48[A]	Estragole	42.42	62.83
	6.48[B]			
3	8.61[A]	α-Cubebene	0.558	0.54
	8.53[B]			
4	9.16[A]	Cubebene	0.093	ND
5	9.54[A]	α-Caryophyllene	0.186	ND
6	10.06[A]	α-Farnesene	0.186	ND
7	10.31[A]	Germacrened D	2.791	4.459
	10.26[B]			
8	11.68[A]	Palmitaldehyde	0.074	ND
9	12.57[A]	11-Dodecen-2 one	1.86	5.945
	12.55[B]			
10	13.67[A]	9,12-Octadienal	2.605	0.202
	13.62[B]			
11	15.06[A]	Tridecanoic acid, methyl ester	5.47	ND
12	16.69[A]	Linoleic acid, methyl ester	12.058	1.451
	16.65[B]			
13	16.75[A]	Linolenic acid, methyl ester	5.917	ND
14	17.03[A]	Linolelaidic acid, methyl ester	0.402	ND
15	19.75[A]	9,12-Octadecadien-1-ol,	0.322	3.243
	19.69[B]			
16	20.73[A]	9,12,15-Octadecatrien-1-ol	0.167	ND

Note: RT: retention time; A = acetone extract; B = ethanolic extract

Another area of interest for the avocado seed is its starch content. In wet basis, **Table 8** shows that the second largest component of avocado seed after moisture is starch at approximately 30%. This opens a wide variety of uses for the avocado seed, as starch can be utilised as a thickening, stabilising, and gelling agent. Also, it can be made into a biodegradable packaging material (M. Lubis et al., 2017). Chel-Guerrero, Barbosa-Martín, Martínez-Antonio, González-Mondragón, and Betancur-Ancona (2016) reported that avocado starch has similar functional properties to commercial maize starch. Despite the avocado seed possessing a lower amylose content than maize, avocado seed starch has a slightly lower gelatinisation temperature than maize starch (65.7˚C and 66.3˚C respectively). Tests on pasting and rheological properties at 5% (w/v) total solids, showed that the avocado seed starch has an enhanced structure at ambient temperature. Specifically, it was suggested that the elasticity of avocado seed starch could provide a smooth texture while remaining soft and flexible and low temperatures (50˚C). It would also retain thickening power at high temperatures (95˚C) and maintain high shear values in heating and cooling processes.

Table 8. Proximate composition of avocado seed (Feurte) (Weatherby & Sorber, 1931).

Parameter	Wet Basis (%)	Dry Basis (%)
Moisture	50.4	0.0
Ash	1.3	2.7
Protein	2.5	5
Reducing Sugars	1.6	3.2
Common Sugars	0.6	1.2
Starch	29.6	60
Pentosans	1.6	3.3
Arabinose	2	4.1
Ether Extract	1	2
Fibre	3.7	7.2
Undetermined	5.6	11.3

Utilising the high starch content, Mahawan, Tenorio, Gomez, and Bronce (2015) looked at preparing biscuits from the avocado seed flour. Ash content was shown to affect water absorption and fermentation activity of baked biscuits. Avocado seed flour is considerably higher in ash (2.83%) compared to normal wheat flour (1.8%) (Mahawan et al., 2015). Furthermore, consumers did not prefer higher ratios of avocado seed flour to wheat flour as it imparted a bitter taste. This bitter taste can be attributed to the presence of polyphenolic content, such as catechins, hydroxycinnamic acids, quercetin, chlorogenic acids, and procyanidins A and B. (Ma et al., 2014; Trujillo-Mayol et al., 2019; W. Wang et al., 2010; Yepes-Betancur et al., 2021). Similarly, another study looking at using a single screw extrusion system to convert avocado seed into a snack found similar issues. However, the authors found that applying heat through extrusion and drying decreased the concentration of tannins (Olaeta, Schwartz, Undurraga, & Contreas, 2007).

With an increasing consumer demand for new and natural colourants, studies suggest that avocado stone can be extracted as a natural orange dye with application in different areas such as cosmetics or food. Dabas, Elias, Lambert, and Ziegler (2011), noticed that avocado seed when crushed with water, developed an orange colour in a time dependant manner. Heat treatment prevented colour development whereas exogenous polyphenol oxidase (PPO) restored colour development. A reduction in phenolic content was observed during colour development, indicating that the polyphenols in avocado seed extract may be oxidised by the PPO. The pigments were stable in a solution (pH 7.5) at -18˚C for up to 2 months. This novel compound was later named perseoranjin but is now commonly referred to as perseorangin. Perseorangin has been identified as a glycosylated benzotropone-containing compounds with a molecular formula of $C_{29}H_{30}O_{14}$ (Hatzakis et al., 2019; Shegog, 2015). Interestingly, perseorangin has also shown potential use as a functional ingredient or used in pharmaceutics due to its anti-inflammatory and anti-cancer activity. The coloured avocado seed extract

(CASE) has shown to reduce viability of human breast (MCF7), colon (HT29), lung (H1299), and prostate (LnCaP) cancer cells *in vitro* (Dabas, Elias, Ziegler, & Lambert, 2019; Dabas, Ziegler, & Lambert, 2019).

Similar to avocado skin, the avocado seed has revealed good properties as an adsorbent material (Elizalde-González et al., 2007). In a recent study, dried avocado seed milled (particle sizes between 0.15-0.5 mm) were used to remove toxic heavy metals such as total chromium and Cr(VI), which were dependent on the pH shift (Aranda-García & Cristiani-Urbina, 2019). Moreover Díaz-Muñoz, Bonilla-Petriciolet, Reynel-Ávila, and Mendoza-Castillo (2016) showed that treating avocado seed with sulfuric, citric and tartaric acids can improve the sorption of heavy metal ions (Cd^{2+}, Cu^{2+} , Ni^{2+} , Pb^{2+} and Zn^{2+}) from aqueous solutions. An increase of up to 600% in metal ion uptake was seen using acid treated avocado seeds compared to untreated avocado seeds. The maximum sorption capacities of acid treated avocado seeds ranged from 3.3 to 21.8 mg/g with the highest removal obtain for Pb^{2+} ions. A. B. Leite et al. (2018) applied conventional pyrolysis to produce activated carbons. Activated carbon prepared at higher temperatures of pyrolysis (700˚C) had the best sorption capacity to up taking emerging organic contaminants.

3 Chapter 3: Converting industrial organic waste from the cold-pressed avocado oil production line into a potential food preservative

3.1 Introduction

One-third of the world's food production for human consumption is either lost or wasted from farm to fork. In many cases, 39% of food losses occur during the manufacturing and processing of food products. These nutritious suboptimal foods are considered undesirable by the consumer, based on sensory and visual deviations. As a result, the by-products are rarely used after disposal (Raak, Symmank, Zahn, Aschemann-Witzel, & Rohm, 2017). In recent years, cold-pressed avocado oil (CPAO) production is on the increase and resulted in rapid market expansion. The use of this oil has been popularised due to its oxidative stability at high temperatures, similar to olive oil (Costagli & Betti, 2015).

The avocado (*Persea americana Mill.*), '*Hass*' cultivar is mostly used as raw material for CPAO, as opposed to other varieties due to its superior yields, fruiting characteristics, and thick skin that protects the fruit during transportation (Wong et al., 2008). CPAO is made up of approximately 10% polyunsaturated fats, 15-20% saturated fats and 60-70% monounsaturates. According to Wong, Requejo-Jackman, and Woolf (2010a), 60-80% of the monosaturated fatty acids in avocado oil is oleic acid. Research suggests that oleic acid can protect against insulin resistance, decrease inflammation in the body and regulate healthy blood lipid profiles (Perdomo et al., 2015a). Avocado oil can also enhance the bioavailability of fat-soluble vitamins and phytochemicals from other fruits and vegetables that are naturally low in fat (Dreher & Davenport, 2013)

Research indicates that avocado oil is abundant in phytochemicals, mainly chlorophylls, carotenoids and α-tocopherol (Wong et al., 2010a). The antioxidative effect of these phytochemicals help to scavenge free radicals, as well as reduce the incidence of various diseases, including age-related macular degeneration (Woolf et al., 2009). A study by Lu et al. (2005) revealed that bioactive carotenoids from avocados, in combination with diet-derived phytochemicals, may contribute to the significant reduction of risk to cancer. Aside from health implications, CPAO is also utilised for culinary purposes as it remains stable at high temperatures with a smoke point of 250˚C, which makes it suitable for shallow pan frying (Woolf et al., 2009). With this notion, the consumer perception of natural products as being less 'polluted', more nutritious and better quality has contributed to its commercialisation.

The process of CPAO (**Figure 8**) first begins with washing the avocado fruit and then drained before de-stoning by separating avocado pulp from the seed and skin. Oil is extracted from the flesh as it goes through a grinder where it is simultaneously crushed and sliced, breaking down cell walls and releasing the oil droplets. Subsequently, the most crucial malaxing stage is where the avocado paste is continuously stirred at a controlled temperature between 40˚C to 50˚C. This technique allows oil droplets to agglomerate, making the downstream separation more efficient. A three-phase decanter then spins this malaxed paste in a horizontal drum separating the pomace (fibrous material), liquid phase (wastewater) and the oil (CPAO). In some cases where a two-phase decanter is used, the liquid phase can be further processed in a centrifuge to remove the final CPAO from the wastewater (Costagli & Betti, 2015; Wong et al., 2008).

Utilisation of the cold-press mechanical extraction method of avocado oil has led to a significant accumulation of by-products, including seed, pomace, peel, and the most abundant being wastewater. These suboptimal parts, especially the skin and seed, contain essential

antioxidants and vitamins are commonly discarded into landfills (Domínguez et al., 2014; W. Wang et al., 2010). As an industry practice, Olivado Ltd, a CPAO processing company based in Kerikeri, New Zealand, uses the pomace as animal feed for bovines. Whereas studies by Saavedra et al. (2017) found that the avocado seed and skin can be turned into powders using a convective drying process, creating powdered storable commodities with high antioxidant activity Moreover, avocado seed has shown potential for use as biofuel due to its high combustible energy content of 19.145 MJ kg^{-1} (Perea-Moreno, Aguilera-Ureña, & Manzano-Agugliaro, 2016).

The disposal of wastewater is problematic for the manufacturer because it cannot be directed into the drains due to its high volume (about 0.45 L wastewater per kg of avocado fruit) and level of organic material. External contractors are usually required to collect and dispose of the wastewater, which is very costly. In this study, the spray drying method will be used to convert the wastewater into avocado wastewater (AWW) powder, which could potentially be used as a functional food ingredient. Spray drying is ideal for processing the wastewater because of its efficient single step evaporation of the fluid material into powder with a reproducible particle size (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). This is the first time that such work has been investigated.

The aims of this study were to investigate the amount of waste or by-products generated as a result of CPAO production and to find an alternative use for the wastewater. Proximate analysis on all four by-products from the CPAO process (seed, skin, pomace and wastewater) were investigated. The spray dried wastewater powder was analysed in terms of particle size, yield, total phenolic content, antioxidant content, and colour. Finally, the feasibility of incorporating this powder into pork sausages as a natural preservative to prevent lipid oxidation was explored.

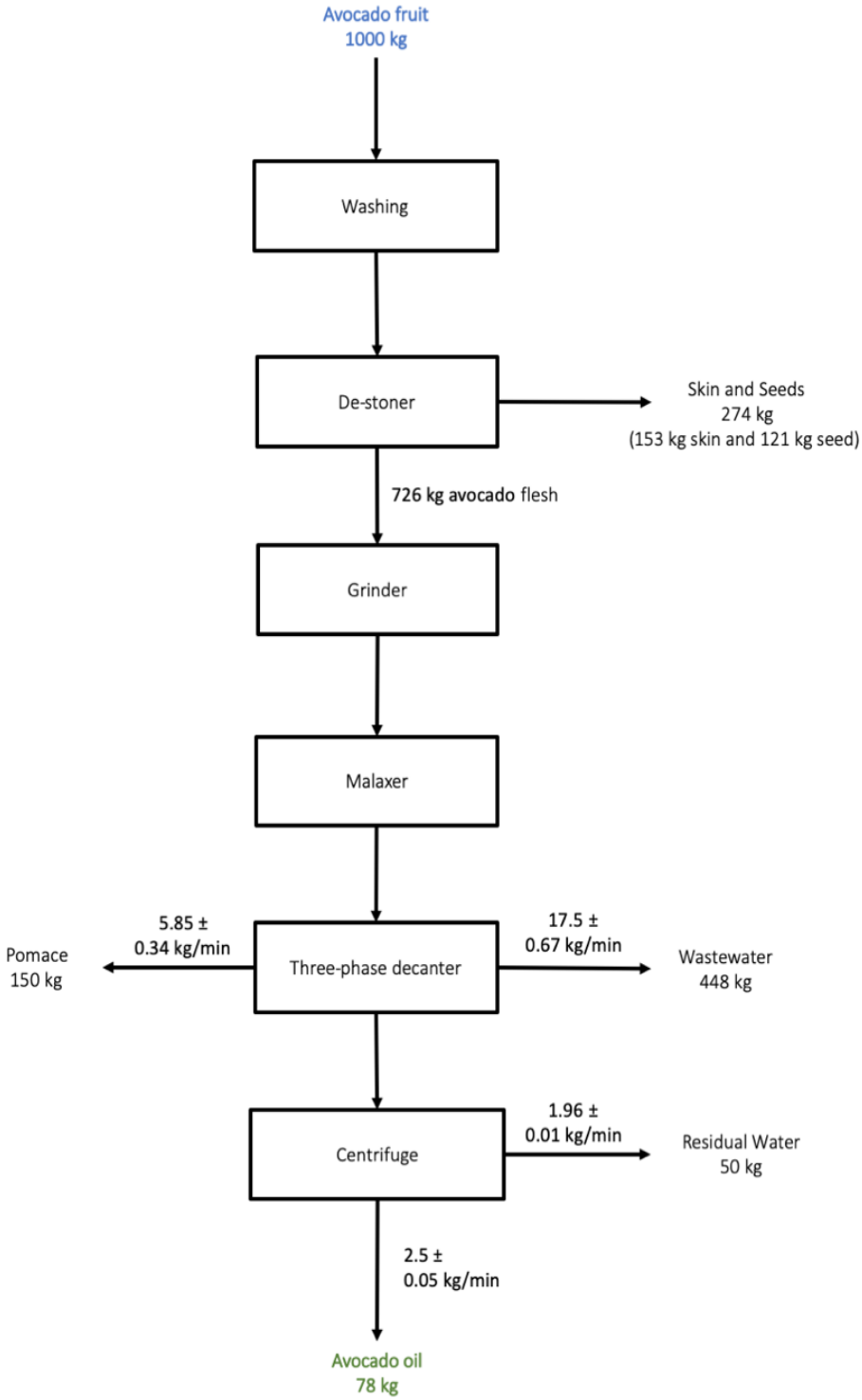


Figure 8. Process flow diagram showing the extraction of cold pressed avocado oil in New Zealand's Kerikeri production line with a three-phase decanter system.

3.2 Materials and Method

3.2.1 Materials

3.2.1.1 Fresh avocado fruits and percentage dry matter

The Orangewood orchard in Northland, New Zealand supplied fresh *Hass* avocado fruits to Olivado NZ Ltd's for cold-pressed oil extraction in late October 2018 (early season). The fruits were held at 20°C in wooden crates to ripen. Two experienced assessors employed by Olivado NZ Ltd used industry standard tactile assessments to determine fruit ripeness (A. White, Woolf, Harker, & Davy, 1999). The avocado fruit was gently squeezed by the assessor's hand to determine the firmness and hence ripeness. Twenty-five (n=25) ripened avocado fruits were selected at random from the wooden crates for proximate analysis. To determine the percentage dry matter, thirty unriped avocado fruits were selected at random from multiple crates. The skin and seed are removed and the remaining flesh dried at 65°C until constant weight (Gamble et al., 2010). The percentage dry matter of these early season avocados were found to be 24%.

3.2.1.2 Collection of waste samples

Avocado wastewater, seed, skin and pomace from *Hass* variety were collected from the Olivado Ltd oil processing plant in triplicates on three separate production days during the week. The seeds and skins were collected directly from the destoner in 26cm x 38cm snap lock plastic bags (Glad, Australia). The pomace was obtained from the decanter. Samples were immediately stored at 4˚C in an ice bath and transported within 3.5 hours to the laboratory at Auckland University of Technology. Wastewater was stored in 5 L PET plastic containers and refrigerated before further use. A portion of the fresh samples were used for proximate analysis while another portion were freeze-dried using the Alpha 1-2 LDplus

Laboratory Freeze Dryer for 48 hours at -75°C and 1 x 10^{-3} mbar and then stored at -18˚C until needed for chemical analysis.

3.2.1.3 Preparation of pork sausages

Pork belly was purchased from Countdown, a local supermarket in New Zealand. Pork belly was the meat of choice for sausages because of its high fat content. The pork belly was minced using a Kenwood Pro 1400 mincer and equally divided into three batches (450 g). Batch 1 was used as a control with 4% (w/w) sodium chloride (NaCl) added to optimise the water holding capacity of the meat (Bernthal, Booren, & Gray, 1989) to make the sausage juicier. Batch 2 contained 4% NaCl and 0.04% sodium erythorbate (E316), a common synthetic antioxidant (preservative) for processed meat products. FSANZ (2016) states that 0.04% (*w*/*w*) of E316 is the maximum allowable limit to be added into meats. Therefore, batch 3 using AWW was based on the cupric ion reducing antioxidant capacity (CUPRAC) equivalence to E316. Batch 3 comprised of 4% NaCl and 0.2% AWW powder, spray dried at 160˚C. Each batch was separately mixed, cased in commercial hog casing (Dunninghams, NZ), and baked at 180˚C for 20 minutes using a Piron PF7005D oven (Italy). Samples were then freeze-dried separately, ground into powder using a Sunbeam AutoGrinder II EM0420, and stored at -18˚C before further analysis.

3.2.1.4 Chemicals

Neocuproine, ascorbic acid, ammonium acetate, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), iron (III) chloride hexahydrate, propyl gallate, sodium carbonate (Na_2CO_3), TBA (2-thiobarbituric acid), TEP (1,1,3,3-tetraethoxypropane) and gallic acid were purchased from Sigma-Aldrich. Acetic acid, hydrochloric acid (37%), and petroleum ether (boiling point: 40-60˚C) were obtained from Fisher Chemicals. Sodium acetate trihydrate was purchased from

LabServ, UK. TBA (trichloroacetic acid), Na_2-EDTA (ethylenediaminetetraacetic acid disodium salt dehydrate), and Folin-Ciocalteu phenol reagent were sourced from Scharlau, Spain. Copper (II) chloride dihydrate was purchased from VWR International, US. Monopotassium phosphate from Interchem. Sulphuric acid (98%) was purchased from Labchem, US. Ammonium molybdate was from Univar, US. Potassium sulphate was provided by ECP Labchem, NZ and copper (II) sulphate was purchased from Merck, US.

3.2.2 Methods

3.2.2.1 Cold-press extraction of avocado oil and measuring avocado waste outputs

Yang et al. (2018) reported the processing steps and operating conditions for CPAO production. Ripened avocado fruit (1000 kg) were supplied to the Olivado Ltd processing plant, Kerikeri, New Zealand. The avocado skin and seeds were collected from the destoner (Alfal Laval, Sweden) in 200 L plastic bins and weighed using an industrial platform scale (± 5 kg) (WS-701, Wedderburn). The avocado pomace and wastewater outputs from the decanter were determined using a 30 L bucket and a timer. Mass flow rates of these components were measured by weighing the total mass (kg) accumulated in a bucket after 1 minute. Likewise, the amount of pure avocado oil and residual water were determined using the bucket and timer method. All measurements were replicated three times and the average was taken as the final value. Once the mass flow rates were known, it could be proportioned to work out the total mass output. Cold pressed avocado oil (CPAO) yield (%) was obtained as per the formula below:

$$\frac{a}{(a+p+b)} \times 100 \qquad \textbf{(Equation 1)}$$

Where a is the mass of avocado oil collected, p is the mass of oil lost in pomace, and b is the mass of oil lost in black water.

3.2.2.2 Proximate analysis of avocado by-products

Standard AOAC (1997) methods were used to determine fat (method 920.39), ash (method 925.09), moisture (method 925) and protein (method 954.01), in the avocado by-products. Fat content was determined using petroleum ether extraction according to the Soxhlet principle (Thiex, Anderson, & Gildemeister, 2003) using a Gerhardt SOXTHERM unit. Ash content was calculated from the difference between initial dried and final sample weight after combustion at 550˚C for 5 hours using a retort furnace (Perfect Fire III, HDTP-56-55). Moisture content was measured based on the mass difference of sample after oven drying at 105˚C for 24 h. Protein content was measured using the Kjeldahl method. Approximately 0.5 g of powder was mixed with concentrated sulphuric acid for digestion on a heating block at 420˚C along with potassium sulphate and copper (II) sulphate as catalysts. Once ready, the sample was then distilled using NaOH and H_3B_3O in the Vapodest 450 distillation unit. This unit then automatically titrated the sample using 0.1M of HCl. Total protein was determined using the formula described in the AOAC standard (AOAC, 1997). A nitrogen conversion factor of 6.25 was used, as no specific conversion factor for avocado by-products exists.

Available carbohydrate (ACH) and dietary fibre (DF) were assessed using a kit purchased from Megazyme (K-ACHDF) (Megazyme International Ireland Limited, Bray, Ireland). Due to high fat content of the dried samples (>10%), they were first defatted using the Soxhlet principle as described previously, to ensure both ACH and DF assays presented accurate results.

3.2.2.3 Spray drying

Avocado wastewater (AWW) was spray dried using a small-scale Buchi mini spray dryer B-290, Switzerland, equipped with a 0.70 mm spray nozzle. The AWW was not standardised however proximate analysis showed that the average solid content was 12.7 ± 0.1%. From preliminary tests, it was found that setting the wastewater feed flow rate to a constant 5.8 g per minute, aspiration rate at 37 m^3/h and the atomising air at 49 m^3/h, gave the highest yield of AWW powder. The inlet temperatures were altered from 110˚C to 160˚C at 10°C increments to investigate its effects on yield using **Equation 2**. Outlet temperatures varied between 62˚C to 102 ˚C respectively. The dried powders from the cyclone and collection vessel were further freeze dried to remove residue moisture in the powder and then put into ziplock bags and stored in an airtight plastic container at -18˚C until further analysis. All spray drying experiments were replicated three times.

Yield calculation: $\frac{Powder\ collected\ in\ cyclone\ (g)}{Mass\ of\ total\ solids\ in\ sample\ before\ spray\ drying\ (g)} \times 100$ (**Equation 2**)

3.2.2.4 Antioxidant analysis

Extraction of antioxidants was carried out based on a protocol described by Santo, Nunez, and Moya (2013) with slight adjustments. Samples were homogenised using a T25 digital Ultra-Turrax in 4 mL of 50% methanol solution at 10,000 rpm for 2 minutes and left to stand for 1 hour. Tubes were centrifuged using the Vortex-Genie II at 2500 rpm for 15 minutes at 20°C; the resulting supernatant was then transferred into a 10 mL volumetric flask. This step was repeated using 70% acetone solution, after which the volumetric flask was filled to the 10 mL mark using distilled water. This solution was further diluted 1:10 using distilled water.

Cupric ion reducing antioxidant capacity (CUPRAC) assay was carried out according to the methods and principles detailed by Özyürek, Güçlü, Tütem, Sözgen Başkan, et al. (2011). Sample volume of 1 mL was added into 1 mL of $CuCl_2.2H_2O$ (0.01 M), NH_4AC (1 M, pH 7), neocuproine (0.075 M), and 0.1 ml distilled water to yield a total volume of 4.1 ml. The solution was left to react for 5 minutes at room temperature and its absorbance was measured against a reagent blank (1 mL of neocuproine, $CuCl_2.2H_2O$, NH_2Ac solution and 1.1 mL water) using the GE Ultrospec 7000 spectrophotometer at 450 nm. A standard curve was plotted with Trolox (5 to 170 mg/L^{-1}; $R^2 = 0.998$). The final antioxidant activity of the sample was measured as mg Trolox equivalent (TE)/100 g powder.

Ferric reducing antioxidant power (FRAP) assay was carried out as detailed by Benzie and Strain (1996). The final FRAP reagent was composed of 1 mL TPTZ (0.01 M), 1 mL of $FeCl_3.6H_2O$ (0.02 M) and 10 mL of acetate buffer (0.3 M). A sample volume of 0.1 mL was added to 0.9 mL of distilled H_2O and 2 mL of the FRAP reagent. The samples were left to react for 5 minutes at room temperature and were spectrophotometrically measured against a reagent blank (2 mL FRAP reagent 1 mL H_2O), at 593 nm. Trolox solutions with concentration varying from 5 to 170 mg L^{-1} were used to generate a standard curve with $R^2 = 0.997$. Results were expressed in mg TE/100 g powder.

Phosphomolybdenum assay was performed as outlined by Ivanišová, Kačániová, Petrová, Frančáková, and Tokár (2016). From each extract, 1 mL of sample was mixed with 2.8 mL KH_2PO_4 (0.1 M), 6 mL H_2SO_4 (1 M), 0.4 mL $(NH_4)_6Mo_7O_{24}$ (0.1 M) and 0.8 mL of distilled water in glass vials. Once mixed, the samples were incubated for 120 minutes at 90°C. They were then rapidly cooled and measured against a blank for absorbance spectrophotometrically at 700 nm. Trolox (3 to 390 mg L^{-1}; $R^2 = 0.996$) was used as the standard. Results were expressed in mg TE/ 100 g powder.

3.2.2.5 Total phenolic content (TPC) by Folin–Ciocalteu's assay

The Folin-Ciocalteu's (F-C) assay was carried out as described by Singleton, Orthofer, and Lamuela-Raventós (1999), with some alterations. Sample extraction was carried out the same as antioxidant analysis (section 2.2.4). After extraction, 1 mL of sample or standard was transferred to a glass vial, mixed in with 500 µl of F-C phenol reagent and, after 5 minutes of reaction, 1.5 mL of 20% Na_2CO_3 was added. The mixture was vortexed for ten seconds, covered with aluminium foil and left in the dark at room temperature (22 ± 0.5°C) for 2 hours. Solutions were transferred into cuvettes and read at an absorbance of 765 nm against a water blank. Gallic acid solutions at various concentrations 0-200 mg/L were used for calibration. The TPC of samples were expressed as gallic acid equivalents (mg GAE/g) by means of a standard curve (R^2 = 0.9995).

3.2.2.6 Scanning electron microscopy imaging and colour analysis

Particle morphology for AWW powders was evaluated using a Hitachi SU-70scanning electron microscopy (SEM). The detectors worked at a distance of 15.5 mm (WD = 15.5 mm) from the samples with an accelerating voltage of 5 kV applied for each sample. Sample micrographs were represented by a 20 µm scale. A platinum coating was applied before scanning using the Hitachi E-1045 Ion Sputter. Particle size (µm) of the SEM images were measured using the ImageJ software (version 2.0.0-rc-43/1.52i). Further details regarding colour analysis on the AWW powders are provided in *Supplementary information SI.1 & SI.2.*

3.2.2.7 Lipid oxidation measurement using TBARS

Thiobarbituric acid reactive substances (TBARS) content was determined colorimetrically by the method of (Vyncke, 1970). When lipid oxidation occurs in meat, it results in several

undesirable products, including malondialdehyde (MDA), which is associated with off-flavours and unpleasant smell (Ghani, Barril, Bedgood, & Prenzler, 2017). High levels of MDA indicate a higher degree of oxidation. About 1 g of the sample was homogenised with a 10 mL solution containing 7.5% trichloroacetic acid solution (TCA), 0.1% propyl gallate and 0.1% EDTA-Na_2 for 30 seconds, then filtered using a Büchner funnel and centrifuged at 2000 rpm for 15 minutes. TBA reagent (0.02 M thiobarbituric acid in distilled water) with a volume of 5 mL was added to 5 mL of filtrate in 15 mL falcon tubes. The mixture was vortexed for 1 minute, incubated in a water bath for 40 minutes at 100˚C and then cooled to ambient temperature. The absorbance was measured at 532 nm and 600 nm ($A_{532\ nm}$ - $A_{600\ nm}$) using a spectrophotometer against a blank of 5 mL TCA and 5 mL TBA. This was done to account for turbidity and instrumental error. TBARS was expressed as mg MDA/kg of minced sausages against a standard curve ($y = 1.0507x - 0.0183$, $R^2 = 0.9996$). The percent inhibition of spray dried powder and E316 against TBARS was calculated as:

$$\% \text{ inhibition} = \frac{C-T}{T} \times 100 \qquad \textbf{(Equation 3)}$$

Where *C* is the control and *T* is the antioxidant treatment where either spray dried powder or E316 was added to the sausage fillings.

3.2.2.8 Statistical analysis

Samples were analysed in triplicates where data were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) with Tukey pairwise comparison of means was performed using the XLSTAT software (version 2018.7). A difference of $p \leq 0.05$ was considered significant.

3.3 Results and discussion

3.3.1 Mass balance on the cold-press avocado oil production line

Figure 8 illustrates the CPAO production line employed by Olivado Ltd with mass balance. Although a similar method was employed by Costagli and Betti (2015), Olivado Ltd used a three-phase decanter for separating the pomace, wastewater and CPAO without further treatment of wastewater after the decanting stage. In the Costagli and Betti (2015) study, wastewater was centrifuged to recover a small portion of oil. This step was omitted in the Olivado Ltd's processing line. After extraction of CPAO, Olivado Ltd then sends its CPAO into a primary storage tank where inert nitrogen is sparged into the oil. Sparging removes oxygen that may have dissolved into the oil and delays rancidity of the oil.

The mass balance (**Figure 8**) shows that for every 1000 kg of avocado fruit, around 78 kg or 85 L of CPAO is produced. The most significant mass of waste was attributed to wastewater (448 kg), followed by skin and seeds with combined weight of 274 kg, pomace (150 kg) and residual water (50 kg). Wong, Eyres, and Ravetti (2014a) also reported a general mass balance summary but based on a two-phase decanter system for an avocado oil processing plant. Their report showed that after processing 796 kg of avocado fruit, wastewater amounted to 408 kg, followed by skin and seed (195kg), and pomace (120 kg). Even though the methodology on how the data was obtained was not reported, in both studies the largest by-product by mass was wastewater.

The total oil yield was calculated to be around 67% in this study (**Equation 1**), which is reasonable as Costagli and Betti (2015) reported similar yields of between 57 to 68%. Wong et al. (2014a); Wong et al. (2010a) explained that up to 75% of the oil could be extracted from early-season avocado (samples with 15% oil by fresh weight). However, up to 85% of the maximum oil (22.5% oil by fresh weight) can be extracted from late-season avocados.

The majority of unextracted oil remained in the wastewater, followed by seed, skin and lastly pomace.

3.3.2 Proximate composition in the avocado by-products

Proximate composition of avocado oil by-products is shown in **Table 9**. The results demonstrated that avocado wastewater was the highest in moisture (88.3%) content followed by the pomace (82.8%), skin (75.3%) and seed (57.0%). The fat and ash quantified in wastewater were significantly higher but lowest in protein when compared to the other components. All proximate values were reasonably similar to what has been reported by Morais et al. (2017). In retrospect, A. F. Vinha, J. Moreira, and S. V. Barreira (2013) reported skin and seeds possessed a lower moisture content of 69% and 54% respectively, and higher fat content of 2.20% and 14.7% respectively. This variability in the proximate composition of the avocado constituents could be due to various reasons. Araújo, Rodriguez-Jasso, Ruiz, Pintado, and Aguilar (2018) stated that the constituent and proximate value of avocado is mainly dependent on the variety, ripeness, climate and composition of soil and fertiliser used, all of which would have been slightly different for each sampled batch in the two studies. Samples from the Morais et al. (2017) study were sourced from Brazil, whereas samples from Ana F Vinha et al. (2013) was from Portugal, meaning that both fruits would have been exposed to different environmental conditions. Neither of the studies reported that the harvesting season had a significant impact on proximate composition.

Avocado wastewater as a by-product of CPAO production is a component, which has not been studied in any previous literature. **Table 9** shows that this component contained the most significant percentage of lipid compared to skin, seed and pomace. Research by Wong et al. (2014a) also found that the wastewater component contained the highest amount of lipid compared to the other three by-products. This high level of lipid content can be attributed to

the processing stage when wastewater was first separated in the decanter (**Figure 8**), where some of the oil droplets were too small to be separated. Yang et al. (2018) monitored the avocado oil droplet size with light microscopy over time during the malaxing process. Even after 120 minutes of malaxing at 45 ± 5°C, oil droplet size below 5µm can be seen in the microscopy images, which is difficult to separate in the decanter.

Skin and pomace had a substantial dietary fibre content of 20.1% and 16.4% respectively with very low amounts of available carbohydrates (ACH), similar to other reported studies (Domínguez et al., 2014); Saavedra et al. (2017). On the other hand, the seed contained 31.7% of ACH making it an unconventional, yet viable starch source. Domínguez et al. (2014) explained that despite their high starch content, the seeds have a high polyphenol concentration, which imparts a bitter taste and could be toxic at higher levels.

3.3.3 Spray drying and powder morphology

Figure 8 shows that during the early avocado season, every 1000 kg of avocados used to manufacture CPAO, approximately 448 kg of wastewater was discarded as waste. This makes up to almost half of the total avocado fruit input. So far, no studies have characterised this by-product and investigated its application in food. This study is the first to carry out physicochemical analysis of wastewater, and to convert the wastewater using spray drying into AWW powder. Inlet drying temperatures of 110˚C through to 160˚C produced powders that did not exceed above 32% yield, which was obtained from an inlet drying temperature of 160˚C (**Table 10**). Garofulić, Zorić, Pedisić, and Dragović-Uzelac (2016) explained that a suitable spray drying yield of 50% or higher is deemed commercially viable. Samples from this study were solely spray dried without incorporating any microencapsulation agent leading to powder stickiness and consequently exhibited low yields with high variability. Hence, would not be commercially viable

Table 9. Chemical composition, antioxidant and total phenolic content of avocado by-products and avocado wastewater (AWW) powder

[1]**Proximate analysis on avocado flesh and by-products (dry basis)**

Samples	Dietary Fibre	Available Carbohydrate	Protein	Ash	Lipid
Skin	81.4 ± 2.0	1.2 ± 2.0	8.1 ± 0.4	3.6 ± 0.4	6.9 ± 2.0
Seed	19.5 ± 1.3	69.1 ± 1.1	4.9 ± 0.2	3.7 ± 0.2	3.7 ± 0.7
Wastewater	22.2 ± 3.4	0.9 ± 3.4	10.3 ± 7.7	17.9 ± 2.6	53.8 ± 9.4
Pomace	72.1 ± 1.2	0.6 ± 1.2	12.8 ± 1.3	7.0 ± 0.6	9.3 ± 6.4
Flesh	ND	26.4 ± 2.1	6.0 ± 0.4	6.4 ± 1.6	61.3 ± 3.4

[1]**Proximate analysis on avocado flesh and by-products (wet basis)**

Samples	[2]Quantity Generated (kg)	Moisture Content	Dietary Fibre	Available Carbohydrate[3]	Protein	Ash	Lipid
Skin	153	75.3 ± 0.5	20.1 ± 0.5	0.3 ± 0.5	2.0 ± 0.1	0.9 ± 0.1	1.7 ± 0.5
Seed	121	57.0 ± 0.9	8.4 ± 0.6	29.7 ± 0.5	2.1 ± 0.1	1.6 ± 0.1	1.6 ± 0.3
Wastewater	448	88.3 ± 0.1	2.6 ± 0.4	0.1 ± 0.4	1.2 ± 0.9	2.1 ± 0.3	6.3 ± 1.1
Pomace	150	82.8 ± 0.3	12.4 ± 0.2	0.1 ± 0.2	2.2 ± 0.2	1.2 ± 0.1	1.6 ± 1.1
Flesh	726	76.5 ± 0.5	ND	6.2 ± 0.5	1.4 ± 0.1	1.5 ± 0.4	14.4 ± 0.8

[4]**Antioxidant properties and total phenolic content of freeze-dried avocado by-products and AWW powder**

Samples	CUPRAC g TE/100 g powder	FRAP g TE/100g powder	Phosphomolybdenum g TE/100g powder	TPC g GAE/100g powder
Freeze-dried avocado by-products				
Seed	2.1 ± 0.09^{d,e}	7.7 ± 0.35^{b}	8.1 ± 1.00^{b}	8.1 ± 0.28^{b}
Pomace	7.1 ± 0.10^{c}	4.0 ± 0.01^{d}	6.8 ± 1.90^{b,c}	3.6 ± 0.13^{d}

Table 9 *(continued)*

Skin	7.0 ± 0.12[c]	13.7 ± 0.76[a]	12.0 ± 1.19[a]	13.7 ± 0.40[a]
Flesh	0.5 ± 0.01[e]	0.3 ± 0.02[f]	0.8 ± 0.01[e]	0.6 ± 0.02[e]
Wastewater	3.3 ± 0.87[d]	2.0 ± 0.19[e]	3.2 ± 0.4[d,e]	1.6 ± 0.12[e]
AWW powder				
A (110˚C)	8.8 ± 0.87[b,c]	3.1 ± 0.13[d,e]	5.0 ± 1.18[c,d]	3.9 ± 0.24[c,d]
B (120˚C)	8.0 ± 0.84[c]	3.6 ± 3.60[d]	6.5 ± 0.74[b,c]	4.4 ± 0.38[c,d]
C (130˚C)	11.3 ± 1.36[a,b]	4.1 ± 4.07[d]	7.3 ± 0.31[b,c]	5.1 ± 0.58[c]
D (140˚C)	11.1 ± 0.70[a,b]	4.0 ± 0.33[d]	7.2 ± 0.41[b,c]	4.7 ± 0.86[c,d]
E (150˚C)	12.6 ± 0.52[a]	4.2 ± 4.18[d]	7.4 ± 0.38[b,c]	5.0 ± 0.44[c]
F (160˚C)	11.1 ± 0.96[a,b]	5.4 ± 0.56[c]	7.6 ± 0.47[b,c]	5.1 ± 0.30[c]

[1]Values represent the means of triplicates expressed in percentages (dry and wet basis, % w/w), ± depicts standard deviation of the means.
ND = no data.
[2]Based on 1000 kg of avocado fruit input into a three-phase decanter cold-press avocado oil extraction process.
[3]Samples were analysed in duplicates.
[4]Data are mean ± standard deviation on dry weight basis. Same subscript letters in the column on each parameter do not differ statistically by Tukey's test ($p < 0.05$) where samples were analysed in triplicates. Spray dried powder parameters: see Table 10. Trolox equivalent (TE). Gallic acid equivalent (GAE).

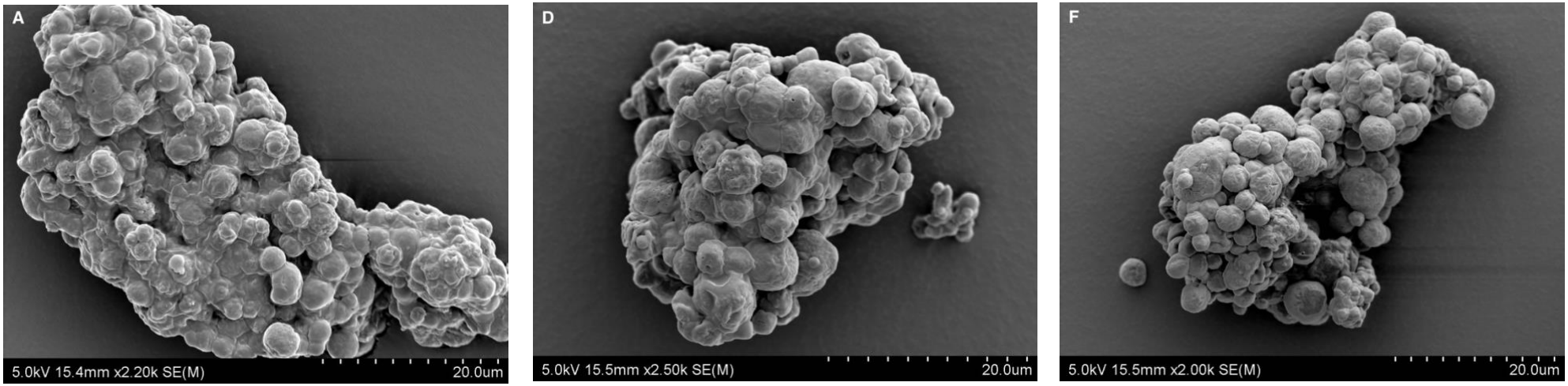


Figure 9. SEM images of AWW powders, spray dried at different temperatures: (A) 110˚C; (D) 140˚C; (F) 160˚C. Samples were randomised images of what was seen under the SEM for each spray drying condition. Note that the whole scale bar is 20.0 µm, and each increment is 2.0 µm .

Stickiness affects yield as most of the spray dried product is attached to the drying chamber and cyclone of the spray dryer. Garofulić et al. (2016) explained that fruit juices usually exhibit stickiness and caking, due to a high amount of low molecular mass sugars such as glucose and fructose, which have low glass transition temperatures. Consequently, this encourages the tendency of the powder to stick the dryer walls leading to low product yield. As avocado samples were higher in fat than sugar, high-fat products can also demonstrate similar sticky issues due presence of low melting point triglycerides as reported by Bhandari, Datta, and Howes (1997). As seen in **Table 9**, it can be seen that the most abundant component after moisture in wastewater were lipids.

Table 9 also showed that wastewater was naturally low in carbohydrates, so it cannot effeciently encapsulate the oils. Higher carbohydrate content provides low viscosity at high solid content and good solubility. However, as carbohydrates have low interfacial properties that are required for increased microencapsulation, gums or proteins are usually incorporated (Gharsallaoui et al., 2007). Chasekioglou, Goula, Adamopoulos, and Lazarides (2017) similarly reported extensively lower yields of about 3% as a result of stickiness while spray drying olive mill wastewater (OMWW). To overcome this, maltodextrin was added as a carbohydrate drying agent at a ratio of 1:1 (OMWW solids)/(drying aid solids) that increased the yield to 23.7%.

SEM micrographs of the spray dried powder are presented in **Figure 9**. All spray dried powders appeared as agglomerates of smaller particles rather than separate individual particles. At lower inlet temperature (110°C), the powders appeared to be tightly gelled together, and the particle morphology was less defined. However, at 160˚C, the agglomerates appeared more morphologically distinguished and separated. **Table 10** shows that the particle size distribution was about the same for Sample A (2.7 ± 0.57μm) and D (2.6 ± 0.21μm) with the most significant difference seen in Sample F (3.5 ± 0.8μm). However, it was evident that

Sample F, with the highest heat application (160˚C), displayed a lower degree of coalescence, resulting in larger, more distinguished particles. This is similar to findings by Dantas, Pasquali, Cavalcanti-Mata, Duarte, and Lisboa (2018) who spray dried avocado pulp mixed with milk sugar and maltodextrin.

When air inlet temperature is low, the evaporation rate produces microcapsules with increased water content, membrane density and poor fluidity. This is depicted in Sample A (110˚C) that exhibited very densely packed agglomerates that slowly start to coalesce together. Alternatively, a high inlet temperature causes rapid moisture evaporation consequently resulting in cracks in the membrane(Gharsallaoui et al., 2007), which is evident in Samples D and F . **Figure 9** shows that the morphology of Sample A is mostly irregular and very rough. With the increase in spray drying temperature, Samples D and F although agglomerated together showed internal spherical shape with an irregular surface and smoother surfaces.

3.3.4 Determination of total phenolic content (TPC)

The avocado skin contained the highest TPC compared to the other CPAO by-products, and the lowest TPC was seen in the pomace (**Table 9**). Fruit peels are known to be rich in polyphenols and antioxidants, as they protect the fruit from oxidative stress caused by sunlight and high temperatures (Mokbel & Fumio, 2005). Total phenolic content in skin, seed and pomace were 13.7, 8.1 and 3.6 g GAE/100 g dried mass (DM) respectively. Kosińska et al. (2012) similarly reported that *Hass* avocado peel contained a higher TPC of 25.3 CE/g (DM) compared to seed (9.5 mg CE/g). In contrast, W. Wang et al. (2010) reported that the highest TPC was observed in avocado seeds, 5.6 g GAE/100g DM, and was far lower in peels, 1.2 g GAE/100g DM. This could be explained by the fact that different extraction solutions were used. W. Wang et al. (2010) used a mixture of acetone, water and acetic acid

at a ratio of 70:29.7:0.3 respectively, while Kosińska et al. (2012) extracted TPC using 80% methanol at a solid to solvent ratio of 1:8, with 2 additional methanol rinses.

Spray dried AWW powder did not differ significantly ($p < 0.05$) in TPC amongst the different drying temperatures. However, there was a slight increase in TPC with increasing inlet temperature. Presumably due to the formation of Maillard reaction products (Dantas et al., 2018) Interestingly, the TPC of spray dried wastewater samples were significantly higher than freeze dried samples as shown in **Table 9**. A study by Soong and Barlow (2004) found a higher phenolic content in heated (140-180˚C) mango seed kernel powder (MSKP) than freeze dried MSKP.

3.3.5 Antioxidant properties

As one protocol is not precise enough to establish the complete antioxidant potential of a natural extract, this study utilised three assays at different pH values to evaluate the by-products of avocado oil production including seed, pomace, skin and wastewater (spray dried). Reducing power of extracts of each by-product was determined using CUPRAC and FRAP assays. CUPRAC involves reduction of Cu (II) to Cu (I), whereas the FRAP method involve reduction of Fe(III) to Fe(II) by antioxidants. The third protocol looked at total antioxidant activity using the phosphomolybdenum assay, based on the reduction of Mo(VI) to Mo(V).

The skin of avocado displayed the highest antioxidant activity in two (FRAP and phosphomolybdenum) out of the three assays carried out. This was expected as several articles similarly concluded that the skin of the avocado to be high in antioxidant activity and reducing ability (Kosińska et al., 2012; Rodríguez-Carpena, Morcuende, Andrade, Kylli, & Estévez, 2011; W. Wang et al., 2010). Nonetheless, the results differed from a study by (Ana Ferreira Vinha et al., 2013)who found that avocado seeds exhibited significantly higher

antioxidant activity compared to the skin of the avocado. Ana F Vinha et al. (2013) concluded that both skin and seed had high antioxidant activities similar to our results summarised in **Table 9.**

Rodríguez-Carpena et al. (2011) reported that TPC and CUPRAC assays resulted in antioxidant activity of peels and seed extracts that were considerably higher than that of avocado flesh extracts. This was also evident in our study as seen in **Table 9,** where CUPRAC and TPC values of flesh (0.5 g TE/100 g and 0.6 g GAE/100 g powder respectively), which were considerably lower compared to avocado skin, seed and spray dried powders. W. Wang et al. (2010) reported that the antioxidant capacity of avocado seeds and peels were several folds higher than those reported for raw blueberry (5.3 mg GAE/g), a fruit which is most popular in the health food community for its high antioxidant capacity. In this study, CUPRAC showed the lowest antioxidant activity for seed (2.1 TE/100 g DM) extracts, and the highest in spray-dried powder extracts (between 8.8 to 12.6 g TE/100 g powders) compared to FRAP and Phosphomolybdenum assays. The reducing ability of the dried powder increased with increasing spray drying inlet temperature similar to TPC results. A study on the effects of spray drying temperatures on lemongrass leaf extracts similarly showed that a gradual increase in spray drying temperature from 110°C through to 150°C led to a significant ($P<0.05$) increase in antioxidant activity (Tran & Nyugen, 2018). The pattern of increased antioxidant activity between lower and higher spray drying temperatures can be seen in the CUPRAC, FRAP and Phosphomolybdenum assays depicted in **Table 9.** Similar results were reported by Dantas et al. (2018) who worked on spray dried avocado pulp. Increasing spray drying inlet temperatures subsequently led to an increase in TPC and antioxidant activity due to the formation of Maillard reaction products that have antioxidant and phenolic activity.

Table 10. Summary of spray drying parameters and colour properties of AWW powder.

	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F
[1] Inlet temperature (˚C)	110	120	130	140	150	160
[1]Outlet temperature (˚C)	60–79	65-85	69-82	84–86	90-96	90-120
[1] Yield (%)	21.1 ± 6.73	19.4 ± 2.96	22.6 ± 6.27	18.56 ± 2.83	18.3 ± 3.07	32 ± 7.86
Particle Size (μm)	2.7 ± 0.57	N.D	N.D	2.6 ± 0.21	N.D	3.5 ± 0.8
[2] **Powder colour properties**						
L*	69.4 ± 5.11	N.D	N.D	69.4 ± 1.66	N.D	64.2 ± 1.37
a*	0.2 ± 0.35	N.D	N.D	0.5 ± 0.10	N.D	2.0 ± 0.70
b*	25.0 ± 0.72	N.D	N.D	27.6 ± 0.70	N.D	30.9 ± 0.51

Data are mean ± standard deviation on dry weight basis.
[1] Spray dried samples were performed as triplicates (n=3)
[2] Samples measured in triplicates (n=3)

3.3.6 TBARS content in pork sausages mixed with AWW powder

Lipids readily undergo oxidation reactions, driven by the degree of its unsaturated fatty acids and the presence of catalysts and molecular oxygen in the environment. Several oxygenated products such as peroxyl and hydroperoxides are generated during lipid oxidation, which potentially leads to more undesirable characteristics in meat products during storage. The thiobarbituric acid-reactive substances (TBARS) assay indicates the level of secondary products produced through lipid oxidation (Alirezalu et al., 2018).

Preliminary analysis using the CUPRAC assay showed that the antioxidant capacity of E316 was five times that of the AWW powder spray dried at 160°C. **Figure 10** presents the degree of lipid peroxidation of free fatty acids using the TBARS test in three different batches of sausages containing the following additives, 4% NaCl (control), 4% NaCl with 0.04% E316, and 4% NaCl with 0.20% AWW powder. The results indicated that both E316 and AWW powder were able to inhibit lipid oxidation because the MDA content was significantly lower than the control. The control sample had the highest level of MDA (0.85 mg MDA/kg of dried sausage meat). This was expected as there were no antioxidants in the sausage to prevent lipid oxidation in the control. On the other hand, the sausage containing E316 and AWW powder yielded 0.67 and 0.68 mg MDA/kg of dried sausages respectively. There were no significant differences between using AWW powder at 0.20% w/w and synthetic E316 at 0.04% w/w *($p<0.05$)*. The ability of AWW powder to prevent lipid oxidation in the sausages would infer that there are lipid soluble antioxidants present in the powder.

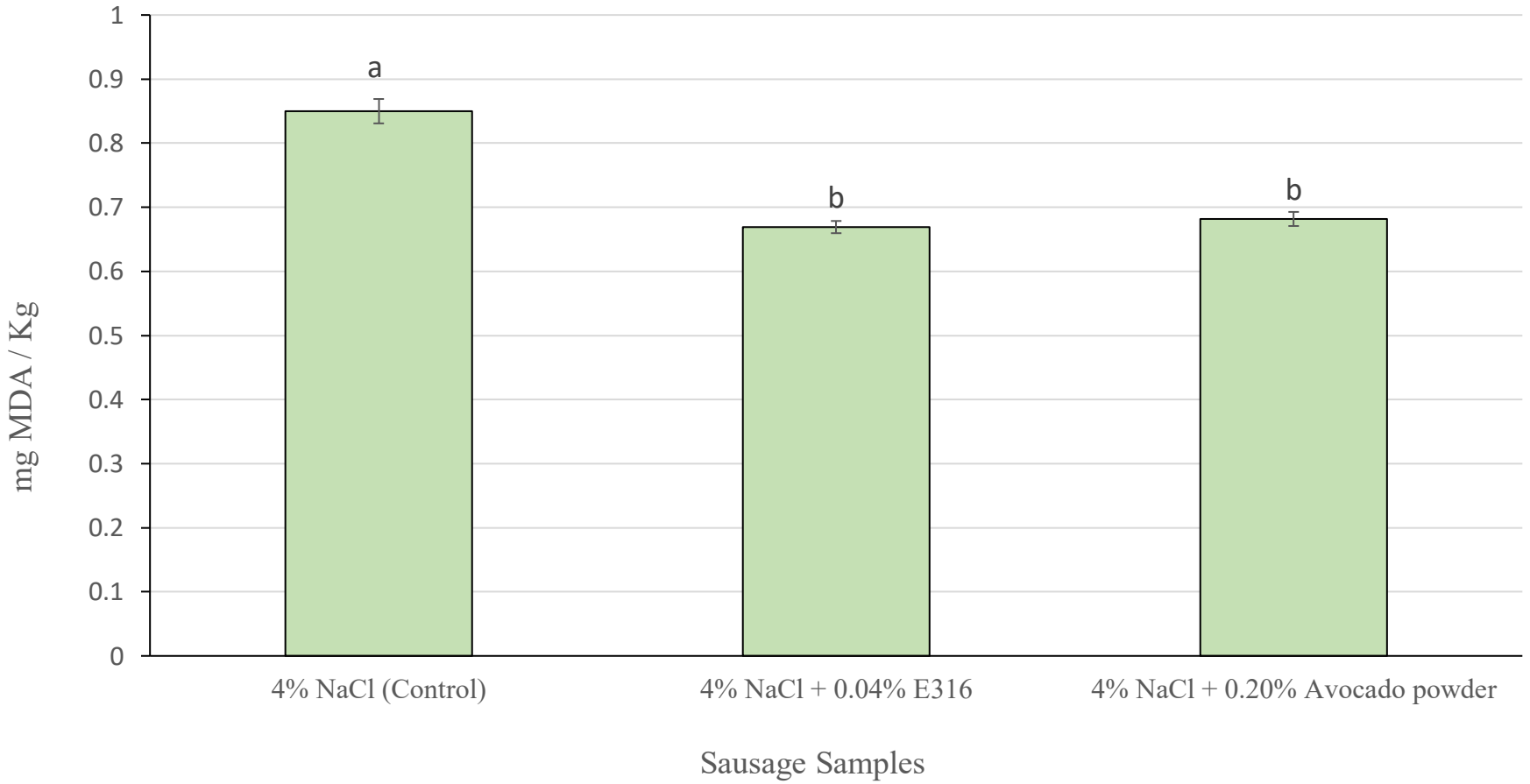


Figure 10. Comparison of spray dried AWW powder against synthetic antioxidant (E316) on TBARS values (mg MDA/kg) in pork sausages. Subscript letters on each parameter do not differ statistically by Tukey's test ($p < 0.05$) of each sample that was carried out in triplicates. Error bars represent the standard deviation of means. Sample F (160˚C) was used in this assay.

A study by Rodríguez-Carpena et al. (2011) utilised the TBARS assay to study the effects of seed and peel extracts from both *Hass* and *Fuerte* avocado cultivars in pork patties. Results from the study concluded that percentage inhibition of avocado extracts ranged from 72.4 to 91.5 %. These values were higher than the current study where percentage inhibition was calculated to be 21.7% (NaCl + E316) and 19.9 % (NaCl + wastewater powder). The significant difference in oxidation inhibition percentages in Rodríguez-Carpena's study could be because TBARS of the patties were measured after being chilled for 15 days at 5˚C, whereas TBARS in this study was assessed after samples were cooked. Additionally, the patties from Rodríguez-Carpena et al. (2011) study used seed and skin waste components, and according to our results these by-products possessed a higher antioxidant activity and reducing ability (**Table 9**), which can explain the higher degree of oxidation inhibition in the pork patties.

3.4 Conclusion

The present work was carried out to evaluate the proximate analysis and antioxidant properties of avocado seeds, peels, pomace and wastewater, which are all by-products from the avocado oil production process. The total mass of by-products constitute approximately 92% of the whole avocado fruit. Avocado seeds were the only by-product that contained a large portion of digestible carbohydrates 29.7% (wet basis) of the whole seed. Furthermore, it was found that wastewater contained the highest lipid content (6.3%) and could be successfully spray dried without the use of carriers. Although the yield was low mainly due to the high lipid content, spray drying was found to increase antioxidant activity, and total phenolic content by 20- and 8-fold respectively compared to avocado flesh. Colour was not influenced much by increasing spray drying temperatures. However, increasing spray drying inlet temperatures resulted in less agglomeration of AWW powders. Antioxidant activities of

seed and peel were high across the CUPRAC, FRAP, phosphomolybdenum and TPC assays, where pomace had the lowest values. The spray dried wastewater powder had the highest antioxidant activity using the CUPRAC assay but had significantly lower FRAP, phosphomolybdenum and TPC antioxidant values compared to seed and skin by-products. Application of the spray dried AWW powder into cooked pork sausages was found to be as effective as using a synthetic antioxidant such as sodium erythorbate (E316) in inhibiting lipid oxidation.

4 Chapter 4: Optimising the Spray Drying of Avocado Wastewater and Use of the Powder as a Food Preservative for Preventing Lipid Peroxidation

4.1 1ntroduction

Demand for avocado (*Persea americana Mill.*) has significantly increased over recent years, due to its health promoting nutrients in the mesocarp. These include saturated, polyunsaturated, and monounsaturated fats; β-carotene; α-tocopherol; and essential minerals such as magnesium and potassium (USDA, 2018). The five largest avocado producing countries, i.e., Mexico, Dominican Republic, Peru, Colombia and Indonesia, have collectively increased their avocado production from 2.4 million to 4.0 million tonnes from 2012 to 2018 (FAO, 2018).

Avocado fruit has applications in the cosmetics and food industries. The use of avocado oil for skin moisturisers and cosmetics have been reported since the 16th century. However, commercialisation of avocado oil for culinary purposes has only been popularised in the past 20 years (Wong et al., 2014a). Previous extraction methods of avocado oil for cosmetics and skin products have utilised harsh solvents technology such as hexane at high temperatures (>60 °C). The extracted oils would then be refined, bleached, and deodorised to remove any undesirable organoleptic properties (Wong et al., 2008; Woolf et al., 2009). Recent advancements in avocado oil extraction have included ultrasound treatment and supercritical CO_2 methods (Corzzini, Barros, Grimaldi, & Cabral, 2017; Martínez-Padilla, Franke, Xu, & Juliano, 2018). Although successful and feasible, these technologies have yet to be commercialised.

Chapter 5

The first successful development of cold pressed avocado oil (CPAO) was initiated in New Zealand and commercialised in 2000. CPAO appeals to consumers because of its attractive emerald colour, buttery flavour, and high smoking point of over 250 °C (Woolf et al., 2009). Consumers also benefit from its oleic acid content and presence of phytochemicals such as carotenoids, chlorophylls, and α-tocopherol. These phytochemicals act as antioxidants, which promote anti-inflammatory actions, regulate healthy blood lipid profiles, and increase bioavailability of fat soluble vitamins (Perdomo et al., 2015b; Unlu, Bohn, Clinton, & Schwartz, 2005).

The CPAO process generates large masses of by-products in the form of seed, skin, pomace, and wastewater. **Figure 11** depicts the major by-products generated from a typical CPAO production line. Using 1000 kg of avocado fruit during the early harvest season as a basis, 12.1% (*w*/*w*) and 15.3% of the input are removed as avocado seed and skin, respectively, during the de-stoning stage. Next, 15% pomace and 44.8% wastewater are removed from the malaxed avocado pulp emulsion at the three-phased decanting stage. The final by-product is removed through centrifugation as 5% residual water, producing a final 7.8% of pure CPAO (Permal, Leong Chang, et al., 2020).

Avocado by-products such as skin and seed can be turned into powders as nutrient-rich storable commodities, using convective drying procedures (Saavedra et al., 2017). Furthermore, avocado seed has shown potential as a biofuel, an alternative source of starch, and a natural colour pigment (Chel-Guerrero et al., 2016; Hatzakis et al., 2019; Perea-Moreno et al., 2016). However, valorisation of the major CPAO by-product that accounts for almost half the mass of avocado fruit input, avocado wastewater (AWW), is somewhat limited. AWW is a major concern in the avocado oil industry as it incurs high disposal costs and cannot be discarded into local drains due to its high organic matter. One way to circumvent this problem is to convert it into a higher value product.

Proximate analysis by Permal et al. (2019) [10] showed that AWW (% dry basis *w*/*w*) was primarily composed of 53.8 ± 9.4% lipids, followed by 22.2 ± 3.4% dietary fibres, 17.9 ± 0.6% ash, 10.3 ± 7.7% protein, and 0.9 ± 3.4% available carbohydrates. The authors successfully converted AWW from a commercial CPAO production line into dried powder with high antioxidants and total phenolic content. Furthermore, they successfully incorporated the powder into pork sausages as a preservative to prevent lipid peroxidation. However, the powder yields from this research were low, ranging from 18.6% to 32%, because the process was not optimised, and no carriers were used. Researchers have found that products with certain sugars or high fat content could result in low powder yield during spray drying due to stickiness from sugar with low T_g (glass transition temperatures) or low melting point triglycerides (Bae & Lee, 2008; Bhandari et al., 1997; Garofulić Ivona, Zorić, Pedisić, & Dragović-Uzelac, 2016). To overcome this, gum, protein, or carbohydrate-based carriers can be added to encapsulate and form a barrier around freely dispersed active material. Microencapsulation through spray drying has proven to be efficient and practical, favouring product quality, increasing shelf life of fruit powders, maintaining stability of bioactive compounds, and increasing powder yield (Dantas et al., 2018; Garofulić Ivona et al., 2016).

Previous research by Permal, Leong Chang, et al. (2020) added spray dried AWW powder in sausages for inhibiting lipid peroxidation and found it to be as good as sodium erythorbate (E316). However, the bioactive component of AWW powder responsible for effective inhibition of lipid peroxidation was not determined in the study. Therefore, to add to the current body of literature, the aims of this study were to increase and optimise AWW powder yield through spray drying and to quantify the fat-soluble antioxidants responsible for preventing lipid peroxidation. Finally, the incorporation of this AWW powder into pure pork

fat as a natural preservative was tested against commercial preservatives commonly used in the food industry.

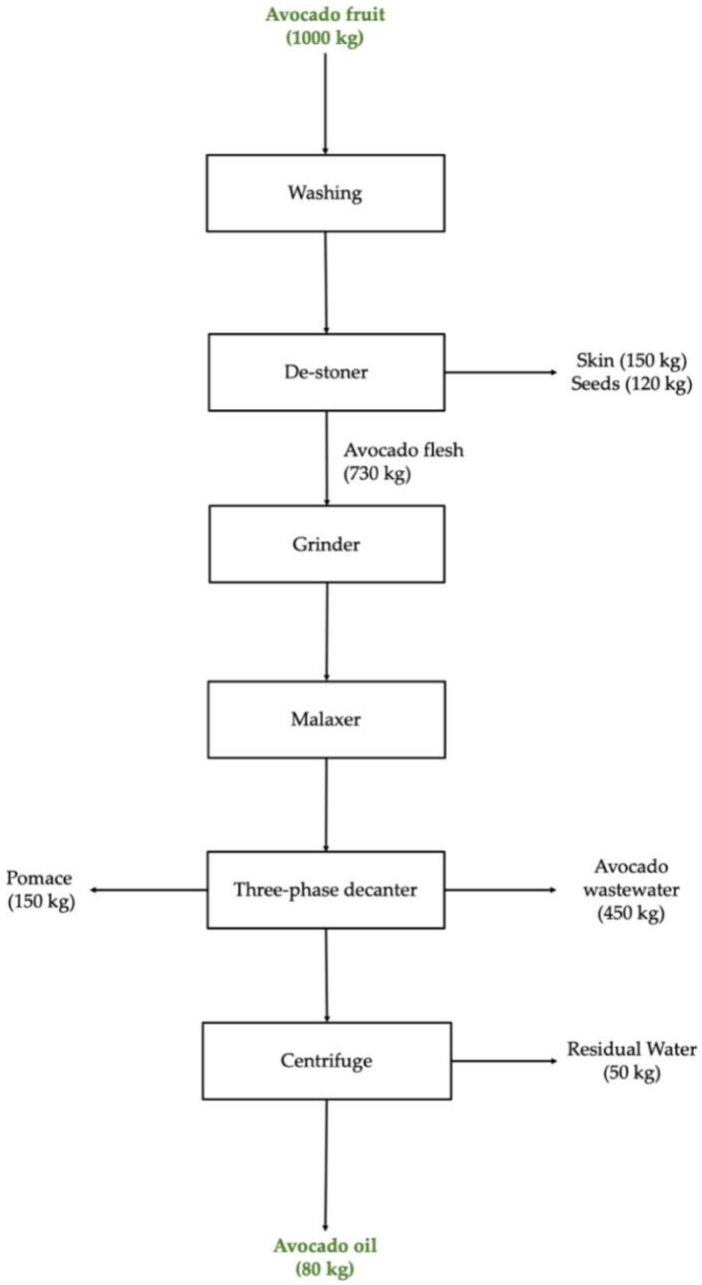


Figure 11. Process flow diagram of a typical commercial cold pressed avocado oil (CPAO) extraction process. All the avocado waste output values from the early harvest season were obtained using an input of 1000 kg avocado fruits as the basis.

4.2 Materials and Methods

4.2.1 Collection of avocado wastewater samples and fresh avocado fruits

Orangewood orchard in Northland, New Zealand supplied the Hass avocado fruits to Olivado Ltd. for commercial extraction of CPAO in late October 2019. The percentage of dry matter of these early season avocados was found to be 24%. The fruits were held at 20 °C inside wooden crates for ripening before processing. AWW was collected from the output of a three-phase decanter (**Figure 11**), on Olivado Ltd.'s CPAO processing line. Three batches of AWW was collected in 5 L PET bottles on three separate production days and immediately stored at 4 °C before spray drying. Additionally, separate fresh samples of AWW were freeze dried using the Alpha 1–2 LDplus Laboratory Freeze Dryer for 48 h, at −75 °C, and 1×10^{-3} mbar, and stored at −18 °C until further analysis.

4.2.2 Chemicals

Neocuproine, ammonium acetate, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), propyl gallate, TBA (2-thiobarbituric acid), TEP (1,1,3,3-tetraethoxypropane), gallic acid, β-carotene, α-tocopherol, and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich, Auckland, New Zealand. Copper (II) chloride dihydrate was purchased from VWR International, Aurora, USA. Chloroform, methanol, hexane, and ethanol were purchased from Thermo Fisher Scientific, Auckland, New Zealand. Butylated hydroxyanisole (BHA) was purchased from BDH chemicals Ltd., Poole, England. Sodium erythorbate (E316) was obtained from D.M Dunningham Ltd., Auckland,New Zealand. Maltodextrin with a 10-12 dextrose equivalence (MD 10–12 DE) and MD 17–19 DE were both purchased from Davis Food Ingredients, New Zealand. Acacia gum was obtained from Hawkin Watts Ltd., Auckland, New Zealand. Lactose was purchased from BDH Chemicals and whey protein concentrate (WPC) sourced from Thompson's, Auckland,

New Zealand. Tri-chloroacetic acid (TCA) and Na_2-EDTA (ethylenediaminetetraacetic acid disodium salt dehydrate) were purchased from Scharlau, Barcelona, Spain.

4.2.3 Spray drying

AWW was spray dried using a laboratory scale Buchi mini spray dryer B-290 equipped with a Buchi B-296 dehumidifier (Switzerland) to remove all moisture in the spray drying air. The spray dryer unit was coupled with a 0.70 mm spraying nozzle. Preliminary tests found that atomising air at 49 m^3/h, and an aspiration rate at 37 m^3/h, were the most suitable parameters for optimal yield of AWW powder without carriers. To prepare each emulsion for drying, carriers were homogenised with AWW using the Silverson L4RT, at 5000 rpm, for 5 min. Four parameters were varied to find the ideal spray drying conditions for the highest yield of AWW powder. Firstly, the addition of different carriers including MD 10–12 DE, MD 17–19 DE, acacia gum (AG), lactose, and whey protein concentrate (WPC) was explored. The AWW feed rate was adjusted, varying between 3 to 11 g/min. The inlet temperatures were varied from 120 °C to 180 °C, at 20 °C increments, and carrier concentration of 1%, 5%, and 10% were used. A higher temperature range was used in this chapter compared to the Chapter 3 (inlet temperature of 160˚C), as adding carriers still produced a great yield. In the previous study, preliminary data indicated that increasing spray drying inlet temperature above 160˚C, without the addition of carriers, resulted in low yield. The basis of selecting these carrier concentrations and spray drying temperatures were based on preliminary tests that provided a suitable range of yields to analyse patterns. All the parameters were collectively investigated for screening purposes to determine their effects on AWW powder yield using **Equation (4**) as described by Permal, Leong Chang, et al. (2020):

$$\text{Yield } \% = \frac{Powder\ collected\ in\ cyclone\ (g)}{Mass\ of\ sample\ before\ spray\ drying\ (g)} \times 100 \qquad \textbf{(Equation 4)}$$

4.2.4 Scanning Electron Microscopy (SEM) Imaging of Spray Dried Powders

Particle morphology for AWW powders using the five different carriers was evaluated as described by Permal, Leong Chang, et al. (2020). The particle size (μm) of SEM images was measured using ImageJ, an open source software (version 1.52u) developed at the National Institutes of Health (NIH), USA.

4.2.5 Extraction and liquid chromatography mass spectrophotometry (LC-MS) analysis of β-carotene and α-Tocopherol

The method to quantify β-carotene and α-tocopherol from AWW using liquid chromatography mass spectrophotometry (LC-MS) has not previously been studied, Therefore, the extraction method used in this research was specifically designed for this purpose. Briefly, 1 g of AWW powder was measured into 15 mL falcon tubes. Then, 0.5 mL of methanol followed by 1 mL hexane were added to the powder. Each solvent addition was vortexed for 30 s. The falcon tubes were, then, centrifuged for 5 min, at 2700 rpm, using the Vortex-Genie II. The resulting top layer of hexane was removed using a glass pipette and dispensed into a 5 mL glass test tube. The extraction using 1 mL hexane was repeated two more times. All the hexane was evaporated using nitrogen. Then, the reduced viscous solution was re-dissolved in 0.2 mL of ethanol, vortexed for 30 s, centrifuged for 5 min at 2700 RPM, and transferred into silanised inserts. The inserts were capped inside 1.5 mL amber vials and immediately analysed in the LC-MS.

Quantification was performed by using commercial β-carotene and α-tocopherol standards to generate a calibration curve in the range of 0.16–20 mg/L (R^2 = 0.999). Chromatographic repeatability (n = 10) was estimated and the residual standard deviation (RSD) was calculated at 6% for α-tocopherol and 11% for β-carotene. Recovery for α-tocopherol and β-carotene was calculated using **Equation (5).** The recovery experiment was carried out by spiking 0.1 g

of powder with 50 uL of 10 mg/L standards and underwent the same extraction method for samples as described above.

$$\text{Recovery \%} = \frac{LC\ MS\ concentration\ \left(\frac{mg}{L}\right)\ reading}{Theoretical\ vitamin\ concentration\ \left(\frac{mg}{L}\right)} \times\ 100 \qquad \textbf{Equation 5}$$

$$\text{LoD} = 3.3 \times \frac{S_{\text{residuals}}}{\text{Slope}} \qquad \textbf{Equation 6}$$

$$LoQ = 10 \times \frac{S_{residuals}}{Slope} \qquad \textbf{Equation 7}$$

Limit of detection (LoD) and limit of quantification (LoQ) were calculated based on **Equations (6)** and **(7)** (Evard, Kruve, & Leito, 2016). $S_{residuals}$, is the residual standard deviation from the calibration curve of compounds in the LoD region, and *slope* is the slope from the calibration curve of each component. LoD and LoQ for α-tocopherol were 0.04 mg/L and 0.13 mg/L, respectively, and for β-carotene, LoD and LoQ were 0.39 mg/L and 1.30 mg/L, respectively.

The LC-MS analysis was conducted using an Agilent 1260 Infinity Quarternary LC System (Santa Clara, CA 95051, USA). The system consisted of the following components: 1260 infinity quaternary pump, 1260 infinity ALS sampler, 1260 infinity TCC column component and a 1260 infinity diode array detector (DAD) connected to a 6420 triple quadrupole LC/MS system with multimode ionisation source. A Waters XSelect CSH C18 (2.1 × 100 mm, 3 µm) column was used for the analysis. The mobile phases were composed of water containing 0.1% (*v*/*v*) formic acid (A) and methanol (B). The initial gradient condition was 5:95 (A:B). From 0 to 1 min, B was increased to 100% and held for 7.5 min,

then, from 8.5 to 9.2 min, B was decreased to 95%. The injection volume was 3 µL and the total run time was 15 min for each sample.

The MS ionisation source conditions were set as outlined: Capillary voltage of 2 kV and corona current of 4 µA, drying gas (N_2) temperature of 300 °C at a flow rate of 5 L/min, vaporiser temperature of 250 °C, and nebuliser pressure of 50 psi were used. The positive ion mode was performed with MRM for quantitative analysis. Precursor-to-product ion transition used for α-tocopherol was, $[M+H]^+$ *m*/*z* 431 → 165,137 with a fragmentor voltage of 100 V and collision energy of 32 eV and 50 eV, respectively. The precursor-to-product ion transition used for β-carotene was $[M+H]^+$ *m*/*z* 537 → 537, 277 with a fragmentor voltage of 160 V and collision energy of 1 eV and 16 eV, respectively.

4.2.6 Preparation of pork fat for lipid peroxidation tests

Pork fat was purchased from a local butcher in Auckland, New Zealand and used to quantify the absolute degree of fat peroxidation using the TBARS (thiobarbaturic acid reactive substances) test. Pork fat was used instead of protein-rich meat, as research has shown that proteins can interfere with TBARS test by giving higher MDA (malondialdehyde) values (Dasgupta & Klein, 2014; Papastergiadis, Mubiru, Van Langenhove, & De Meulenaer, 2012; Tsikas, 2017). To prepare the samples, pork fat was minced using a Kenwood Pro 1400 mincer, and then equally divided into seven treatments. Treatment 1 was used as a control with no additives and Treatment 2 contained 0.04% (*w*/*w*) sodium erythorbate (E316). FSANZ (2016) states that 0.04% (*w*/*w*) of E316 is the maximum allowable limit to be added into meats. Therefore, Treatments 3, 4, 5, and 6 were based on the cupric ion reducing antioxidant capacity (CUPRAC) equivalence of each additive to E316. Treatments 3, 4, 5, and 6 contained 1.5% (*w*/*w*) AWW, 0.1% (*w*/*w*) BHT (butylated hydroxytoluene), 0.01% (*w*/*w*) BHA (butylated hydroxyanisole), and 1.86% (*w*/*w*) α-tocopherol respectively. Treatment 7 was used as a positive control and contained 0.1% (*w*/*w*) WPC. Once prepared, the samples were transferred into glass beakers in triplicate and baked at 180 °C for 15 min using a Piron PF4005D oven (Italy). Then, all samples were cooled to room temperature and immediately analysed for degree of fat oxidation using the TBARS protocol.

4.2.7 Cupric ion reducing antioxidant capacity (CUPRAC) analysis for equivalence of antioxidant activity amongst selected preservatives

The extraction of antioxidants from samples was carried out as reported by (Permal, Leong Chang, et al., 2020). Cupric ion reducing antioxidant capacity (CUPRAC) assay was conducted as detailed by (Apak, Guclu, Ozyurek, & Karademir, 2004). Firstly, 1 mL of appropriately diluted sample, was added into 1 mL of $CuCl_2 \cdot 2H_2O$ (0.01 M), NH_4AC buffer

(1 M, adjusted to pH 7), neocuproine (0.075 M), and then 0.1 mL of distilled H_2O (total volume of 4.1 mL). The sample solutions were held for 5 m at room temperature and absorbance was measured against a reagent blank (1 mL, neocuproine, $CuCl_2 \cdot 2H_2O$, NH_4AC buffer solution, and 1.1 mL water) using a GE ultrospec 7000 spectrophotometer at 450 nm. Concentration was calculated from a Trolox standard curve (5 to 170 mg/L, $R^2 = 0.9965$). The CUPRAC analysis (**Table 13**) indicated that the antioxidant capacity of β-carotene was not significantly different from the AWW powder ($p > 0.05$). However, preliminary TBARS analysis of β-carotene produced MDA values far higher (~6 mg MDA/kg of pork fat) than the control. This could have been attributed to its intense orange pigment interfering with spectrophotometry results. Therefore, β-carotene was not presented in **Figure 14.**

4.2.8 Lipid oxidation of pork fat using TBARS

The Thiobarbituric acid reactive substances (TBARS) value was determined colorimetrically (Permal, Leong Chang, et al., 2020). Therefore, 1 g of pork fat sample was measured out in triplicate, inside 15 mL falcon tube, and mixed with a 5 mL solution containing 7.5% trichloroacetic acid solution (TCA), 0.1% propyl gallate, and 0.1% EDTA-Na_2, along with 5 mL of TBA reagent (0.02 M thiobarbituric acid in distilled water). The tubes were incubated at 100 °C for 40 min and cooled to ambient room temperature in an ice bath. Once cooled, the absorbance was measured at 532 and 600 nm. The extent of peroxidation in terms of malondialdehyde equivalents was determined based on a series of TEP (1,1,3,3-tetraethoxypropane) standards ($R^2 = 0.9996$).

4.2.9 Statistical Analysis

Samples were analysed in triplicate and data were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) with Tukey pairwise comparison of means was

performed using the XLSTAT software (version 2018.7). A difference of $p \leq 0.05$ was significant.

4.3 Results and Discussion

4.3.1 Optimising spray drying conditions

Previous research by Permal, Leong Chang, et al. (2020) on spray dried AWW, exhibited low yields not exceeding 32%, because of powder sticking to the spray drier chamber wall. Proximate analysis from this study revealed that AWW was high in lipid content (53.8 ± 9.4% *w*/*w* dry basis), which could result in stickiness due to the presence of low melting point triglycerides (Bhandari et al., 1997). One way to circumvent this issue was to spray dry AWW using dehumidified air as a drying medium and to encapsulate the avocado oil present in AWW using carriers such as MD 10–12 DE, MD 17–19 DE, AG, lactose, or WPC. Other spray drying parameters including, feed flow rate (g/min), temperature, and carrier concentration were also varied to optimise AWW powder yield.

The optimising process started with the selection of the best encapsulating carrier for AWW during spray drying. Figure 2A shows that the WPC carrier gave the highest average powder yield at 48% followed by lactose at 32%. There were no significant differences *(p > 0.05)* between MD 10–12 DE, MD 17–19 DE, and AG as compared with the control with yields ranging from 19 to 24%. Therefore, WPC was chosen as the carrier of choice for the consecutive spray drying experiments.

Altering feed flow rate (g/min), as shown in Figure 2B, had a significant impact on powder yield ($p < 0.05$) when 5% WPC loading and 140 °C drying temperature were kept constant. The highest average yield was observed at a feed flow rate of 5.8 g/min. Further increase in feed flow resulted in decreased yields. Spray drying of AWW with 5% WPC resulted in an increase in powder yield with an incremental increase of spray drying inlet

temperatures (Figure 2C). The highest average yield of 49% AWW powder was obtained at inlet temperatures of 160 °C and 180 °C. As shown in **Figure 12D**, carrier concentration of 5% WPC resulted in significantly higher ($p < 0.05$) yield than 1% WPC (32%) and 10% WPC (43%).

In this study, WPC resulted in the highest powder yield as compared with other carriers. Proteins have many desirable functional properties as a wall material, including, solubility, ability to interact with water, film formation, emulsification, and stabilization of emulsion droplets. Proteins are effective at encapsulating oils, as they change their structure during emulsification through unfolding and adsorption at the oil water interface. These proteins form resistant multilayers around oil droplets and with the help of repulsive forces they make stable emulsions that are critical for encapsulation purposes (Jafari, Assadpoor, He, & Bhandari, 2008). It is believed that 5% WPC gave a higher yield than 10% due to the concentration effect. Although 10% WPC produced a higher mass of powder collected, the actual concentration of AWW in the powder was less compared to using 5% WPC. Bae and Lee (2008) reported that WPI (whey protein isolate) was more effective in encapsulating CPAO when used alone as compared with the incorporation with maltodextrin at a 1:1 ratio. Similarly, Jimenez, García, and Beristain (2006) reported WPC as a good encapsulating agent for CLA (conjugated linoleic acid). Their study indicated a lower degree of CLA oxidation using WPC as compared with WPC/MD (1:1 ratio of WPC and maltodextrin) or GA (gum Arabic). Du et al. (2014) compared the efficiency of five different carriers to overcome stickiness tendencies of persimmon pulp powder. The study concluded that powder yield with addition of 25% WPC was equivalent to the yield of using 45% MD 14–16 DE, 30% GA, and 30% starch sodium octenyl succinate (SSOS). Comparable results were also reported by Fang and Bhandari (2012) who used maltodextrin and whey protein isolate (WPI) as carriers for spray drying bayberry juice. This study found that 1% WPI was sufficient for powder

recovery (>50%), whereas carrier loading of more than 30% of maltodextrin was required for the same purpose. Du et al. (2014) attributed protein as an effective carrier agent due to the surface activity of protein. The surface activity of a protein allows it to transfer through the interface of a feed solution and rapidly form a film while drying. This film effectively overcomes bonding of consecutive droplets and the stickiness between droplets to the chamber wall.

Figure 12B shows a gradual decrease in AWW powder yield with an increasing feed flow rate. Khalilian Movahhed and Mohebbi (2016) explained that increasing feed flow rate could produce dried samples with higher moisture content as larger feed volumes were being passed over at the same time. Therefore, plasticisers, such as water (T_g = -135 °C), decrease the T_g of atomised feed material, leading to insufficient drying of the powder and lower powder yield (Bhandari et al., 1997).

Figure 12C shows that increasing inlet temperature consequently increases AWW powder yield. Similarly, Fazaeli, Emam-Djomeh, Kalbasi Ashtari, and Omid (2012) reported the positive effects of increasing inlet temperatures (110 °C to 150 °C) on the output of mulberry powder. The authors attributed this higher yield to the greater efficiency for heat and mass transfer processes. Increasing inlet temperatures decreased the chance of inadequately dried powders attaching onto the spray dryer's chamber wall. In contrast, Dantas et al. (2018) concluded that utilising a lower inlet temperature (80 °C) in conjunction with adjusted atomization flow rates, produced higher yields for avocado powder. Dantas et al. (2018) utilised inlet temperatures on the lower spectrum (80–120 °C) as their feed material (avocado flesh and milk) contained considerable amounts of low molecular weight sugars with low T_g, as well as high amounts of fats. Dantas et al. (2018) found that application of inlet temperatures above 80 °C would have resulted in lower powder yield as a result of stickiness with drying temperatures coming closer to glass transition temperatures.

The improved yield increasing from 1 to 5% WPC (32% to 49%, respectively), as shown in **Figure 12D**, was consistent with studies by both Fazaeli et al. (2012) and Dantas et al. (2018). Both studies found that increasing carrier concentrations, significantly increased process yield of black mulberry juice and avocado powder, respectively. The authors reported that increasing carrier concentration increased T_g values of other amorphous fractions present in the mixture which were naturally low in T_g components. Tonon, Brabet, and Hubinger (2008) demonstrated that increasing carrier concentration decreased process yield, which could be due to an increase in mixture viscosity. This may explain the decreased yield in this study when using 10% WPC to encapsulate AWW as compared with 5% WPC.

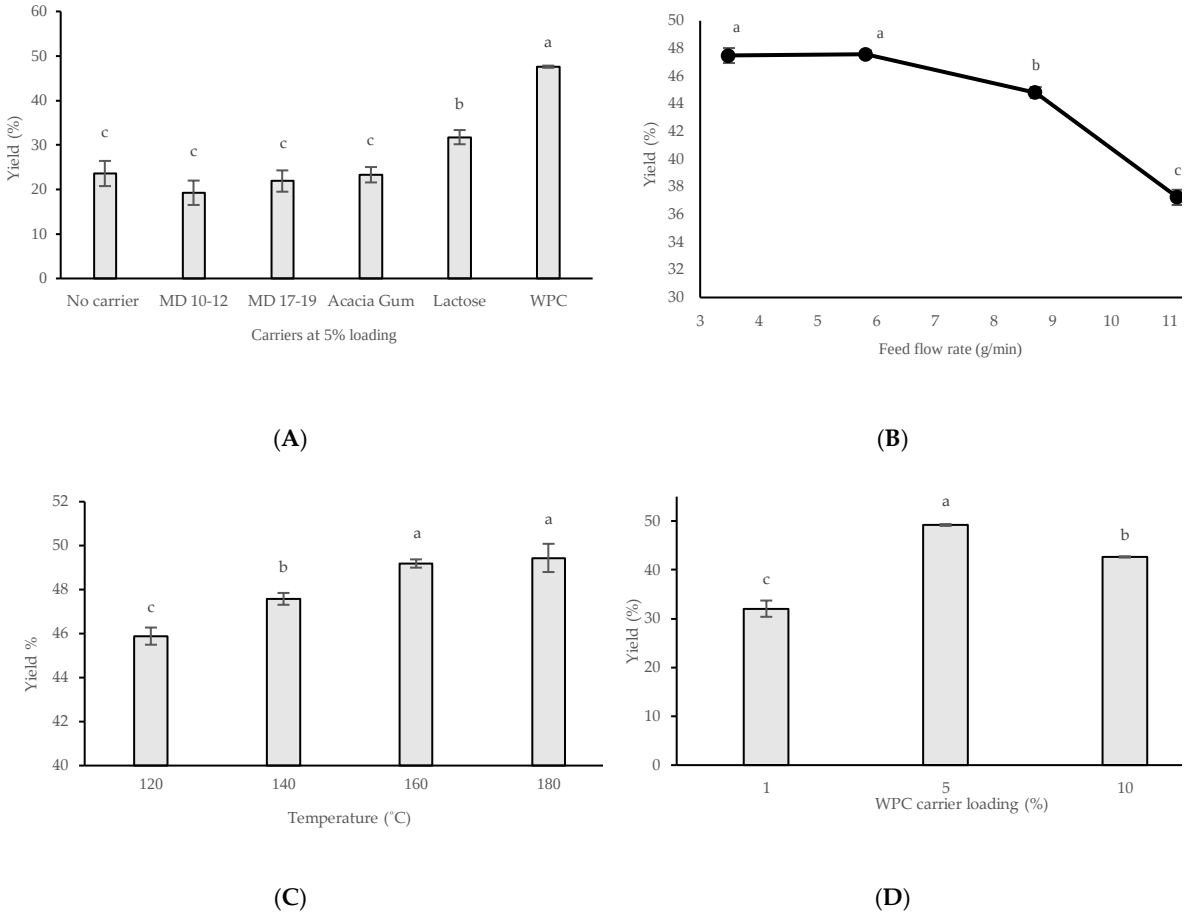


Figure 12. Yield of spray dried avocado wastewater (AWW) under different spray drying conditions. Each data point was measured in triplicate (n = 3) where error bars represent standard deviation of means. (**A**) Effect of carrier types on powder yield when drying temperature of 140 °C, 5.8 g/min feed flow rate, and carrier concentration of 5% w/w with respect to AWW were kept constant; (**B**) Effect of feed flow rate on powder yield when drying temperature of 140 °C and 5% whey protein concentrate (WPC) carrier loading were kept constant; (**C**) Effect of spray drying temperature on powder yield when flow rate of 5.8 g/min and 5% WPC carrier loading were kept constant; (**D**) Effect of WPC carrier loading at a constant feed flow rate of 5.8 g/min and drying temperature of 160 °C. Superscript letters that are the same do not differ statistically based on Tukey's test ($p < 0.05$).

4.3.2 Powder morphology for spray dried AWW powder

AWW powder samples in **Figure 13B–F** were produced with a feed flow rate of 5.8 g/min, 5% (w/w) carrier loading, and spray drying inlet temperature of 140 °C. The SEM analysis of spray dried AWW encapsulated with MD 10–12 DE (4.3 ± 2.36 μm), MD 17–19 DE (4.6 ± 1.75 μm), AG (4.6 ± 3.39 μm), lactose (4.14 ± 1.41 μm), and WPC (4.4 ± 2.23 μm)

showed little difference in average particle size. Morphology of freeze-dried AWW (**Figure 13A**) was the most unique as compared with those spray dried with carriers. The powder appeared as, agglomerated, irregular flake particles. The highest degree of polymerisation of encapsulated powders was seen in lactose (**Figure 13E**) because of its low T_g, 101 °C (Bhandari et al., 1997). However, AG, MD 10–12 DE, MD 17–19 DE, and WPC were in a mass of spherical agglomerates.

Previous SEM micrographs of spray dried AWW powder without encapsulation appeared as agglomerates of smaller particle sizes (<4 μm), rather than separate individual components (Permal, Leong Chang, et al., 2020). In the present study, increasing inlet temperature resulted in a lower degree of coalescence, thus, producing distinguishable spherical particles. With the addition of carriers, spray dried AWW particles still appeared to be agglomerated, but the degree of particle separation and spherical morphology were higher. There was low particle size variability between MD 10–12 DE and MD 17-19 DE (**Figure 13B,C**). However, Tonon, Brabet, and Hubinger (2010) found that açai powder particles produced with MD 10 DE exhibited a median diameter higher than MD 20 DE and AG. They reported that the increase in particle size was influenced by the molecular size of each carrier agent.

Spray dried AWW powder had a high degree of particle agglomeration, making it difficult to distinguish individual particle sizes (**Figure 13B–F**), possibly due to the presence of surface fat bridging between particles. Surface topology of powders, as shown in **Figure 13B–F**, indicated the presence of some pores, cracks, and surface depression. Kim, Chen, and Pearce (2009) explained that quick formation of powder crusts could cause surface fissures or breakages. Alternatively, if the microcapsule crust is moist and supple, the particle could deflate and shrivel upon cooling.

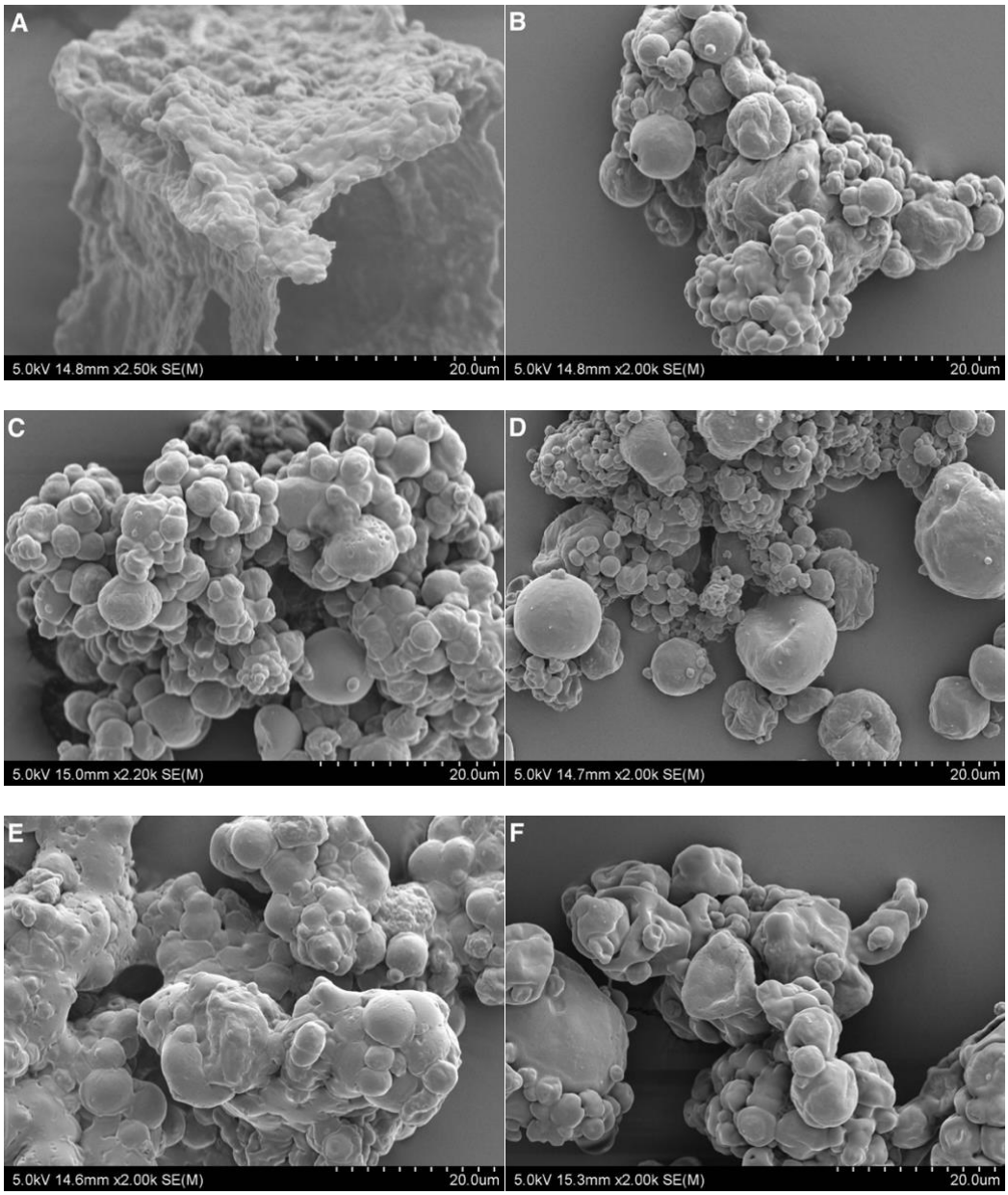


Figure 13. SEM images of AWW powders. The following data represents powder treatment as well as mean particle size ± standard deviation of powder. (**A**) Freeze-dried AWW, 1.57 ± 0.29 µm; (**B**) Maltodextrin with a 10-12 dextrose equivalence (MD 10–12 DE), 4.3 ± 2.36 µm; (**C**) MD 17–19 DE, 4.6 ± 1.75 µm; (**D**) Acacia gum, 4.6 ± 3.39 µm; (**E**) Lactose, 4.14 ± 1.41 µm; (**F**) WPC, 4.4 ± 2.23 µm. Samples were randomised images of what was seen under the SEM for the various parameters. Samples (B) to (F) were subjected to spray drying conditions of 140 °C, 5.8 g/min feed flow rate, and carrier concentration of 5% *w*/*w* with respect to AWW.

4.3.3 Quantifying total β-carotene, α-tocopherol content in AWW powder using the LC-MS

In a previous study, spray dried AWW powder was found to be rich in antioxidants, more so than freeze-dried avocado flesh (Permal, Leong Chang, et al., 2020). The powder

was successfully added into pork sausages as a natural preservative and showed no significant differences in terms of lipid peroxidation as compared with use of the synthetic E316 (sodium erythorbate) preservative. Analysis of antioxidants were conducted spectrophotometrically providing total antioxidant capacity and activity. However, research from Permal, Leong Chang, et al. (2020) did not identify compounds contributing towards the inhibition of lipid peroxidation. Wong et al. (2010a) stated that CPAO contained high concentrations of naturally occurring antioxidants, specifically α-tocopherol (70–190 mg kg^{-1} oil) and carotenoids (1.0–3.5 mg kg^{-1} oil). Yang et al. (2018) reported that not all avocado oil droplets were removed from AWW during CPAO production, which suggested that the preservative properties of AWW could be largely influenced by the presence of carotenoids and tocopherol.

The LC-MS analysis carried out in this study focused on total β-carotene and α-tocopherol content in AWW powder because of their solubility in lipids. This is the first time such an analysis has been conducted on spray dried AWW. The current extraction method was relatively efficient in recovering α-tocopherol (93.4%) (**Table 11**). The powder samples which were analysed included those spray dried with carriers, freeze-dried avocado flesh, and neat AWW. As shown in **Table 12**, avocado flesh had the highest concentration of α-tocopherol (278.7 ± 11.76 mg α-tocopherol/kg powder), nearly triple the amount of what was present in freeze-dried AWW. Interestingly, freeze-dried AWW showed no statistical difference to AWW powders encapsulated with lactose, acacia gum, MD 10–12 DE, and MD 17–19 DE ($p > 0.05$). AWW encapsulated with WPC was the only spray dried sample that had significantly higher α-tocopherol content as compared with freeze-dried AWW. This demonstrated the ability of WPC to maintain and protect α-tocopherol from degradation. Powders dried at 110–160 °C (**Table 13**) without the addition of carriers exhibited a higher concentration of α-tocopherol than encapsulated samples (**Table 12**). This is due to the

concentration effect of carriers, as addition of carriers would have increased solid content of the sample without increasing phytochemical content.

Table 11. LC-MS recovery of α-tocopherol and β-carotene.

Active Component	Initial Concentration (µg/g)	[1] Amount Spiked (µg)	Expected LC-MS Reading (µg/g)	Actual LC-MS Reading (µg/g)	[2] Recovery (%)
α-Tocopherol	1.0	0.5	1.5	1.4	93.5 ± 3.1
β-Carotene	0.2	0.5	0.7	0.1	18.0 ± 8.5

[1] Concentration of spiked α-tocopherol and β-carotene. [2] Data is mean ± standard deviation (n = 3).

Table 12. Total α-tocopherol and β-carotene content of freeze-dried and spray dried AWW and avocado flesh.

Samples	mg α-Tocopherol/kg Powder	[3] mg β-Carotene/kg Powder
Freeze-dried samples		
[1] Flesh	278.7 ± 11.76 [a]	8.6 ± 1.02 [b]
[1] AWW	99.7 ± 16.81 [c]	2.5 ± --[c]
Spray dried AWW		
[2] Carrier Material		
WPC	181.6 ± 32.24 [b]	15.1 ± 0.23 [a]
Lactose	131.4 ± 21.67 [b,c]	0.0 [c]
Acacia gum	108.1 ± 4.02 [c]	0.7 ± --[c]
MD 10–12 DE	95.5 ± 9.72 [c]	0.0 [c]
MD 17–19 DE	115.4 ± 13.39 [c]	1.5 ± -- [c]

Data are mean ± standard deviation on dry weight basis, all sample analysis was performed in triplicate (n = 3). Superscript letters within the columns for α-tocopherol and β-carotene do not differ statistically using the Tukey's test ($p < 0.05$). [1] Samples were freeze dried. [2] Powders used were spray dried with 5% carrier loading, an inlet temperature of 140 °C, and a feed flow rate of 5.8 g/min. [3] Due to low β-carotene recovery, some triplicate samples only produced one reading. Hence standard deviation was not available for these.

Table 13. Total α-tocopherol and β-carotene content of spray dried AWW without the addition of carriers.

Samples	mg α-Tocopherol/kg Powder	mg β-Carotene/kg Powder
110 °C	187.9 ± 23.09 [a, b]	5.0 ± -- [a]
120 °C	226.8 ± 23.44 [a, b]	8.6 ± 4.83 [a]
130 °C	201.0 ± 33.57 [a, b]	3.4 ± 6.19 [a]
140 °C	186.3 ± 44.77 [b]	4.3 ± 3.81 [a]
150 °C	196.9 ± 23.7 [b]	0.4 ± 0.14 [a]
160 °C	320.2 ± 51.09 [a]	5.0 ± 3.83 [a]

Data are presented as mean ± standard deviation values based on dry weight basis. Analysis of samples was performed in triplicate ($n = 3$). Superscript letters within the columns for α-tocopherol and β-carotene that are the same indicate that mean values do not differ statistically using the Tukey's test ($p < 0.05$).

Spray drying temperatures from 110 °C to 150 °C showed no statistical differences in terms of α-tocopherol content ($p > 0.05$) of AWW powder. Furthermore, the highest inlet temperature of 160 °C produced AWW powder with a significantly higher ($p < 0.05$) α-tocopherol content (320.2 ± 51.09 mg α-tocopherol/kg powder) than AWW powders spray dried at 140 °C and 150 °C WPC (186.3 ± 44.77 and 196.9 ± 23.7 mg α-tocopherol/kg powder, respectively). However, due to the high variability in α-tocopherol content, results utilising an inlet temperature of 160 °C was not significantly different from 110 °C, 120 °C, and 130 °C treatments (187.9 ± 23.09, 226.8 ± 23.44, 201.0 ± 33.57 mg α-tocopherol/kg powder, respectively).

The LC-MS recovery protocol for β-carotene was relatively low (18%, **Table 11**) which consequently produced inconsistent data for total β-carotene in AWW powder, as shown in **Tables 12** and **13**. Nonetheless, the LC-MS analysis in **Table 13** shows no statistical differences for β-carotene content in powders dried between 110–160 °C. **Table 12** shows that WPC retained a significantly higher ($p < 0.05$) concentration of β-carotene (15.1 ± 0.23 mg β-carotene/kg powder) as compared with AWW encapsulated with lactose, acacia gum, MD 10–12 DE, and MD 17–19 DE (0.0, 0.7 ± --, 0.0, 1.5 ± -- mg β-carotene/kg powder). The

results showed that WPC was the most efficient carrier for retaining both α-tocopherol and β-carotene content in AWW powder. Several reports have suggested that protein was an ideal carrier for preserving nutraceutical components of powders due to its excellent film forming ability. The high recovery of powder and α-tocopherol retention with only 5% of WPC could be due to surface active properties of WPC in solutions. Fang and Bhandari (2012) found that proteins migrated towards the air and water interphase, reduced surface fat composition of droplet particles, and consequently decreased exposure of the oils to the temperature extremities in the drying chamber. Secondly, the migration of protein onto the surface of sprayed dried powder was capable of rapidly forming a very thin protein rich film. This protein film could have a higher $T_{g,}$ allowing it to remain in the glassy state and protecting the oils from attaching onto the hotter surfaces of the spray dryer. Moreover, a study by Dian, Sudin, and Yusoff (1996) found that maltodextrin and sodium caseinate retained higher levels of carotene in encapsulated palm oil than a blend of maltodextrin and acacia gum.

Utilising the highest spray drying temperature of 160 °C did not degrade α-tocopherol concentration in AWW powders without carriers. Similarly, Permal, Leong Chang, et al. (2020), reported that increasing the spray drying inlet temperatures (110–160 °C) for AWW without carriers either increased or maintained the level antioxidant activity for AWW. Sabliov et al. (2009) reported that α-tocopherol was stable at high temperatures in the absence of oxygen, but under normal atmospheric conditions the rate of degradation for α-tocopherol increased with increasing temperatures. They showed a reduction in α-tocopherol under atmospheric pressure at temperatures ranging from 40 to 180 °C and concluded that increasing temperature not only decreased α-tocopherol content but also increased the rate of decrease, i.e., 55% of α-tocopherol had degraded after 2 h at 180 °C, and almost 80% of free α-tocopherol reduced after 5 h of exposure. There was no difference observed at temperatures below 120 °C, whereby only 30% of free α-tocopherol degraded after 5 h. Contrary to

holding times reported for α-tocopherol degradation, spray drying is an efficient system for delivering powders straight into the collection vessel without being held at high inlet temperatures for a long time. Hence, the degradation of α-tocopherol should be minimal. Even so, this increase in α-tocopherol could be a result of rapid crust formation at the surface of the powder, forming a barrier to protect the α-tocopherol rich oils at the particle center (Bae & Lee, 2008; Permal, Leong Chang, et al., 2020).

Similar to the stability of α-tocopherol, altering the spray drying inlet temperature did not have a significant impact ($p > 0.05$) on the concentration of β-carotene in spray dried AWW. In contrast, Khalilian Movahhed and Mohebbi (2016) reported a sharp decrease in β-carotene content for carrot celery juice when increasing spray drying inlet temperatures (120–170 °C). Interestingly, the authors reported that increasing carrier loading (10% to 30% maltodextrin) decreased β-carotene content. However, the addition of 5% WPC as a carrier in the current study significantly increased retention of β-carotene (15.1 ± 0.23 mg β-carotene/kg powder, $p < 0.05$) as compared with spray dried powder without carriers (0.4 mg β-carotene/kg powder at 150 °C). Compared to lactose, AG, MD 10–12 DE, MD 17–19 DE, and AWW powders without encapsulation, WPC's rapid film forming capabilities provided superior protection for β-carotene.

4.3.4 Inhibition of lipid peroxidation using AWW powder

Lipid peroxidation is a major cause of deterioration in fat rich foods, especially those containing polyunsaturated fats (PUFAs). Sodium erythorbate, BHT, BHA, and α-tocopherol are common lipid soluble antioxidants used by food industries to prevent lipid oxidation in foods. The CUPRAC assay was carried out on four other synthetic additives to match their antioxidant effect to E316. These included β-carotene, BHT, BHA, and α-tocopherol. **Table 14** shows the mg Trolox equivalent/100 g of powder for each additive, as well as their

equivalent concentration needed to produce an antioxidant capacity, similar to 0.04% of E316. The antioxidant capacity of BHA was significantly higher ($p < 0.05$) as compared with the other six additives and approximately 150 times more potent than AWW powder. **Figure 14** shows that adding 1.50% AWW was just as effective as 0.04% E316, 0.10% BHT, 0.01% BHA, and 0.20% α-tocopherol in inhibiting lipid peroxidation. There were no statistical differences in mg MDA/kg of pork fat after cooking the fat with different antioxidants at 180 °C for 15 min. Interestingly, α-tocopherol was not significantly different from the control ($p > 0.05$) with respect to preventing lipid oxidation. Permal, Leong Chang, et al. (2020) reported that AWW powder was an alternate antioxidant additive, effective in preventing fat oxidation in pork sausages. The study also found that there were no significant differences in levels of MDA complexes formed using 0.20% *w*/*w* AWW without carriers and 0.04% E316 in the sausages. In contrast, **Table 14** shows a higher equivalent of AWW (1.5% *w*/*w*) required to match E316 (0.04 *w*/*w*). The higher concentration of AWW is likely due to the concentration effect of WPC, increasing non-phytochemical concentration of AWW powder produced in the current study.

Table 14. Trolox equivalence of additives added to pork fat determined by cupric ion reducing antioxidant capacity (CUPRAC) assay.

Additives	mg Trolox eq./100 g Powder	% *w*/*w* in Pork Fat
[1] E316	83724 [b]	0.04
[2] AWW	2233 [e]	1.50
BHT	35095 [c]	0.10
BHA	330787 [a]	0.01
α-Tocopherol	16745 [d]	0.20
β-Carotene	1803 [e]	1.86
[3] WPC	0 [f]	0.10

Sample analysis was performed in triplicate ($n = 3$). Superscript letters within the columns for α-tocopherol and β-carotene that are the same indicate that the mean values do not differ statistically using the Tukey's test ($p < 0.05$). [1] E316 was used as baseline for all other additives. The percentage of additives added into pork was calculated as its CUPRAC equivalence to 0.04% E316. [2] Powder used were spray dried with 5% WPC at 160 °C inlet temperature and, feed flow rate of 5.8 g/min. [3] Positive control.

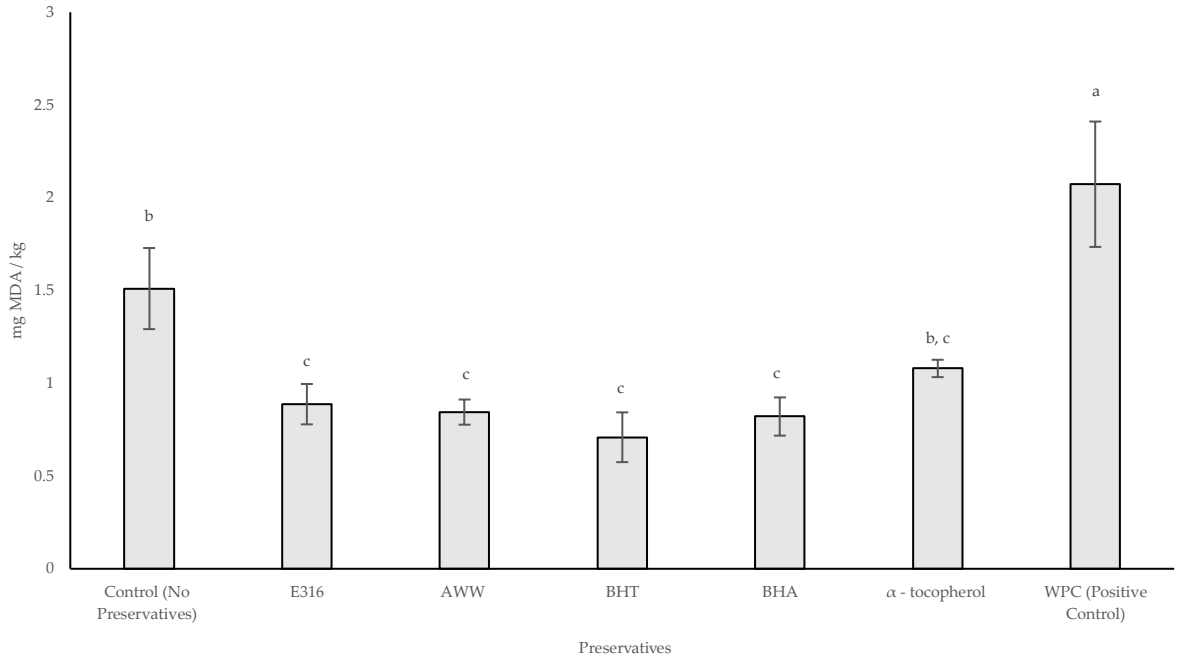


Figure 14. Thiobarbaturic acid reactive substances (TBARS) values (mg MDA/kg) in cooked pork fat containing various synthetic and commercially available additives and spray dried AWW powder. Superscript letters that are the same indicate that mean values do not differ statistically when using the Tukey's test ($p < 0.05$). The analysis of each sample was conducted in triplicate. Error bars represent the standard deviation of means. AWW powder used was spray dried at 160 °C at a flow rate of 5.8 g/min and encapsulated with 5% WPC.

Of the four antioxidants studied, pure α-tocopherol was not as effective in preventing lipid oxidation compared to the control (no preservatives). Ottaway (2008) explained that α-tocopherol was readily oxidised by air. It is heat-stable in the absence of oxygen, but if heated in the presence of oxygen it will degrade faster. The results of this study showed that WPC was able to protect α-tocopherol from degradation during high temperature processing of AWW. The encapsulation of AWW using WPC could preserve the effectiveness of α-tocopherol as an antioxidant, as shown in T**able 12**. However, previous research has shown that proteins could interfere with the TBARS assay (Dasgupta & Klein, 2014; Papastergiadis et al., 2012; Tsikas, 2017). Therefore, the WPC powder which was used as a positive control for the TBARS test was shown to increase MDA content (**Figure 14**). However, when AWW

was encapsulated with WPC, there was no interference with MDA formation due to the low concentration of WPC in AWW as compared with the positive control.

4.4 Conclusion

The design of this study was aimed at optimising existing spray drying conditions of AWW to increase AWW powder yield. The highest yield (49%) was obtained using 5% WPC as a carrier, feed flow rate of 5.8 g/min, and an inlet temperature of 160 °C. The SEM images revealed that the addition of carriers was beneficial in terms of powder morphology. There was a lower degree of particle agglomeration with encapsulation. Encapsulation using WPC was efficient in protecting α-tocopherol and β-carotene. The addition of spray dried AWW into cooked pork fat was as effective as synthetic additives such as BHT, BHA, E316, and α-tocopherol in preventing lipid oxidation.

5 Chapter 5: Converting avocado seeds into a ready to eat snack and analysing for persin and amygdalin

5.1 Introduction

Extruded snacks are popular globally due to their convenience, and their sensory and textural characteristics. According to Chadha, Young, Otter, and Kam (2021), extruded snacks represent up to 50% of the entire ready to eat (RTE) snacks market. Nevertheless, these snack products tend to be energy-dense with poor nutritional value as the ingredients mostly comprise of starch, sugar, fat, and have high sodium content. With the rise in cardiovascular diseases, obesity, and malnutrition, government initiatives have looked to incentivise consumers towards the purchase of nutrient-dense foods.

A possible approach to increase the nutritional quality of such RTE foods in an economical way is to utilise food by-products. By-products from food production can be categorised as (1) agricultural by-products during harvesting, (2) postharvest by-products, and (3) food processing by-products. Agricultural by-products include inedible parts of the plants such as branches and leaves that are not suitable for the food market. Postharvest by-products include produce that do not meet consumer preference based on visual appearance, such as a bruised apple or an undersized potato. By-products from the food processing industry include fruit pomace, seeds, and liquid waste that are removed during processing and before consumption of the product.

Production of cold-pressed avocado oil (CPAO) of the popular ‘Hass’ *Persea americana* Mill variety results in large amounts of by-products such as avocado pomace, skin, seed and avocado wastewater (AWW) as reported by Permal, Leong Chang, et al. (2020). The pomace by-product is used as animal feed for cattle. The avocado skin has been shown to contain

high matrix metalloproteinase inhibitory capacity and antioxidant activity . Permal, Chang, et al. (2020) have shown that AWW can be spray dried into a powder with high phenolic activity. Additionally, the powder was found to be effective in preventing lipid peroxidation in foods.

Avocado seeds have various applications. Hatzakis et al. (2019) isolated perseorangin, a natural colourant from the avocado seed for potential use as dye. Perea-Moreno et al. (2016) successfully converted avocado seeds into biofuel with upper and lower heating values of 19.145 MJ kg^{-1} and 17.889 MJ kg^{-1} respectively. However only a small body of research has focused on the extraction of nutraceutical components such as phenolic acids, procyanidins dimer B and trimers A and B, catechin, perseitol, catechin and epicatechin from avocado seeds through fermentation and microwave assisted techniques (Araújo et al., 2020; Yepes-Betancur et al., 2021).

Due to the high carbohydrate content of avocado seeds, a study by Rivera–González, Amaya–Guerra, and Rosa–Millán (2019) explored the chemical composition and in vitro digestion characteristics of avocado seed flour. After processing, the avocado flour had a yield of approximately 46% which was composed of 27% starch, 6.7% protein, 3.4% fat and 2.71% ash content. Further, *in vitro* starch digestion experiments showed that 56.8% of the carbohydrates in avocado seed flour can be digested. Proximate analysis of avocado seeds, based on dry weight % (DW) by Permal, Leong Chang, et al. (2020) showed that the seed was composed of 19.5% dietary fibre, 69.1% carbohydrates, 4.9% protein, 3.7% ash and 3.7% lipid. The macronutrient profile of avocado seed suggests it would be a suitable raw ingredient to convert into extruded snacks because of its low lipid and protein composition along with its high starch content (Camire, 2001).

Extrusion is a process that combines mixing, shearing, cooking, puffing, final shaping and drying into one low-cost continuous process that can be used to produce a wide variety of

starchy foods. These foods include RTE confectionaries, cereals, and snacks (Yağcı & Göğüş, 2008). With health-conscious consumers keen on purchasing nutritious food products, improvised extrusion technology can be applied to minimise nutritional loss of RTE snacks. Traditional extrusion cooking heats the raw material by methods such as steam injection and heat transfer through water/steam heating jackets surrounding the barrel. An emerging technology in this field is friction cooking, a technique that simply cooks the raw material, solely based on the heat generated through friction between the ingredients and the metal barrel. Some raw materials extruded through the Zapmill™ Friction Cooker include oats, buckwheat, quinoa, tapioca, rice, amaranth, wheat and maize, (Chadha et al., 2021).

Since avocado seeds are not a common food ingredient for human consumption, food safety is an important consideration. Persin (**Figure 20),** a fungicidal toxin**,** is found in idioblast oil cells present in avocado fruit and leaves and is thought to function as a natural insecticide and fungicide (Butt et al., 2006). It has long been known that when lactating livestock consume avocado foliage, they can suffer from non-infectious mastitis and agalactia. However, research has also found that persin may inhibit the growth of certain types of breast cancer cells (Butt et al., 2006; Oelrichs et al., 1995).

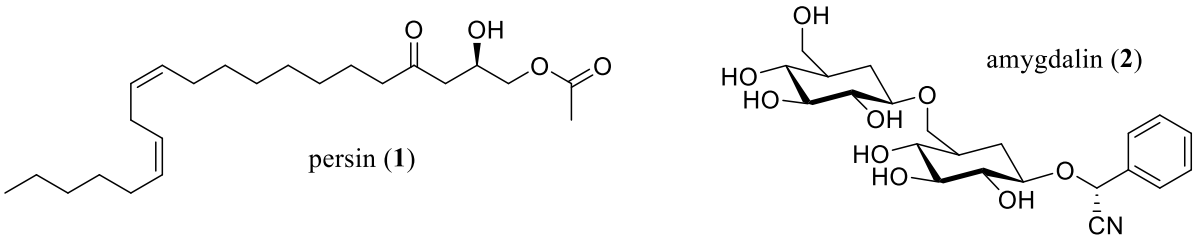


Figure 15. The structures of persin, a toxin found in the avocado plant, and amygdalin, a toxin found in the seeds of many plants.

Amygdalin (D-Mandelonitrile-β-gentiobioside) is a cyanogenic glycoside found in plant seeds. Although cyanogenic glycosides are non-toxic, they become toxic when the plant

tissue is damaged through bruising or chewing as an onset of reactions take place. First the cyanogenic glycoside comes into contact with plant enzymes such as β-glucosidases and α-hydroxynitrile lyases, resulting in cleavage of the carbohydrate moiety from cyanogenic glycoside. Then cyanohydrins are created, which further decompose to release hydrogen cyanide (HCN) along with an aldehyde or ketone (Bolarinwa, Orfila, & Morgan, 2014). The aim of this study was to determine the physical characteristics of RTE extruded snacks made from avocado seed, malted barley, and brown rice using a friction cooker under the same processing conditions. The antioxidant capacities of the three RTE snacks, as well as freeze dried and oven dried avocado seeds were compared. In addition, the amygdalin and persin content in the avocado seed and extruded avocado seed product were determined to confirm their safety prior to consumption.

5.2 Materials and Method

5.2.1 Chemicals

Neocuproine (98%), sodium carbonate (Na_2CO_3), gallic acid, diethyl ether, ammonium acetate (98%), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), iron (III) chloride hexahydrate, sodium carbonate (Na_2CO_3), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97%), and gallic acid were purchased from Sigma Aldrich. Acetic acid, dichloromethane (CH_2Cl_2) and hydrochloric acid (37%) were obtained from Fisher Chemicals. Sodium acetate trihydrate was purchased from LabServ, UK. Folin-Ciocalteu phenol reagent were sourced from Scharlau, Spain. Copper (II) chloride dihydrate was purchased from VWR International, US. Monopotassium phosphate was purchased from Interchem, New Zealand. Sulphuric acid (98%) was purchased from Labchem, US. Ammonium molybdate (81-83%) was purchased from Univar, US. Chloroform, acetone, methanol, and ethanol were purchased from Thermo

Fisher Scientific, New Zealand. Ethyl acetate (EtOAc) was purchased from Macron Fine chemicals, South Africa. D-Amygdalin (98%) was purchased from Alfa Aesar, US. All reagents used were above 99% purity, except for the Folin-Ciocalteu phenol reagent, which is a mixture of several components.

5.2.2 Preparation of raw materials

Approximately 1.5 kg of avocado seeds and leaves were obtained from Olivado Ltd, New Zealand in late October 2019 (early season). Avocado leaves were used in this study to extract persin as a standard, as persin was not available to purchase as a standard. Apricot seeds were packed by Waitaki Orchards Ltd, and purchased from Bidfood, a food wholesaler in Dunedin, New Zealand. Apricot seeds were used in this study as a comparison as they are known to contain a significantly high levels of amygdalin. a Brown rice was purchased from Davis Trading Co, New Zealand. Barley (Gladfield Pilsner malt) was purchased from Home Brew West, New Zealand.

Apricot seeds, as well as avocado seeds and leaves were freeze-dried using the Alpha 1‑2 LDplus Laboratory Freeze Dryer for 48 h at −65 °C and 1×10^{-3} mbar. Another group of avocado seeds were oven-dried (70 °C) using the Piron PF4005D oven (Italy), until the seeds reached a consistent weight (approx. 3 days). Apricot seeds, avocado leaves, and a portion of both freeze-dried and oven-dried avocado seeds were finely ground and stored in the freezer (–20 °C) before analysis. The remaining portion was crushed using a grain mill grinder with roller gap set at 5 mm for friction cooking or stored at –20 °C until required.

5.2.3 Making extruded RTE snacks using the friction cooker

Brown rice, barley, freeze-dried and oven-dried avocado seed were extruded using a single screw, Zapmill™ Friction Cooker (Auckland, NZ) through a 5mm die opening (refer to

Supplementary Information S2.2 for a visual representation). The feed flow rate was fixed at 5.3, 13.1 and 12.8 kg/h for avocado seeds, brown rice, and barley respectively. The screw shaft rotation speed was maintained at 50 Hz. The extruded snacks were air-dried at room temperature for 30 minutes to prevent them from sticking together and stored in airtight containers. The heat generated from friction cooking can elevate the temperature to 130 °C at the exit of the barrel.

5.2.4 Physical analysis of extrudates

5.2.4.1 Lateral expansion ratio

Lateral expansion (LE) ratios were calculated as outlined by Ainsworth, İbanoğlu, Plunkett, İbanoğlu, and Stojceska (2007). Three lengths of extrudate were selected randomly. The diameter of extrudates were measured at 3 different positions along the length of each of the samples, using a vernier calliper. The lateral expansion was calculated using **Equation (8)** below:

$$LE = \frac{diameter\ of\ extrudate - diameter\ of\ die\ hole}{diameter\ of\ die\ hole} \times 100 \qquad \textbf{(Equation 8)}$$

5.2.4.2 Bulk density

Bulk density of the RTE products was calculated by measuring the dimensions of the extrudates (Yağcı & Göğüş, 2008). The lengths and diameters of each extrudate were measured using a vernier calliper. The weight per length of extrudate was determined by weighing measured lengths (1 cm). Assuming a cylindrical shape of extrudate, bulk density was then calculated using the following formula:

$$\rho_b = \frac{4}{\pi d^2 l} \quad \textbf{(Equation 9)}$$

where ρ_b is the density (g/cm^3), *d* is the diameter of the extrudate (cm) and *l* is the length per gram of extrudate (cm/g). An average was taken from five randomly selected extrudate samples.

5.2.4.3 Apparent density

Extrudates were finely blended using a magic bullet blender (MBR-2101) and then sieved through a 500 μm mesh sieve. A 5 mL graduated measuring cylinder was tared on a balance and filled with the extrudate. The bottom of the cylinder was gently tapped on a firm surface until there was no reduction of sample volume and weighed. Apparent density (ρ_s) of extrudates were calculated as mass per unit volume (g/cm^3) for an average of three measurements (Yağcı & Göğüş, 2008).

5.2.4.4 Porosity

Porosity of extruded samples were determined from the apparent volume and bulk volume of the sample, using the following formula (Yağcı & Göğüş, 2008).

$$\text{Porosity} = \frac{bulk\ volume - apparent\ volume}{bulk\ volume} \quad \textbf{(Equation 10)}$$

$$\text{Where: Bulk volume} = 1/\rho_b \quad \textbf{(Equation 11)}$$

$$\text{and Apparent volume} = 1/\rho_s \quad \textbf{(Equation 12)}$$

5.2.4.5 Textural measurements

The textural characteristics of extrudates, specifically, hardness and crispiness, were measured using a Stable Micro Systems TA.XT*plus* textural analyser (Surrey, UK), fitted with a Three Point

Bend Rig (HDP/3PB). The instrument was fitted with a 5 kg load cell. The three-point bending rig comprised of a fixed support with an adjustable separation set to 54 mm. Cross head speed was 0.5 mm/sec and the probe distance was 5 mm. Each extrudate was approximately 70 mm in length.

5.2.5 Antioxidant analysis

Extraction of antioxidants from samples were carried out as outlined by Permal, Leong Chang, et al. (2020). The extracts were analysed for antioxidant capacity using the cupric ion reducing antioxidant capacity (CUPRAC) assay, as detailed by Özyürek, Güçlü, Tütem, Başkan, et al. (2011). Sample extract of 1 mL was mixed into a solution of 1 mL $CuCl_2 \cdot 2H_2O$ (0.01 M), 1 mL NH_4OAc (1 M, pH 7), 1 mL neocuproine (0.075 M) and 0.1 mL distilled water. The solution was left to react for 5 minutes at ambient temperature. The sample's absorbance was measured against a reagent blank (1 mL $CuCl_2 \cdot 2H_2O$, 1 mL NH_4OAc, 1 mL neocuproine and 1.1 mL of distilled water) using the GE Ultrospec 7000 spectrophotometer at 450 nm. A Trolox standard curve was plotted (5 to 86 mg/L^{-1}: $R^2 = 0.9999$). The antioxidant activity of samples was presented as mg trolox equivalent (TE)/100 g powder.

Phosphomolybdenum assay was performed on the sample extracts as outlined by Ivanišová et al. (2016). From each extract, 1 mL of sample was mixed with 2.8 mL KH_2PO_4 (0.1 M), 6 mL H_2SO_4 (1 M), 0.4 mL $(NH_4)_6MO_7O_{24}$ (0.1 M) and 0.8 mL of distilled water. The samples were then incubated for 120 minutes at 90 ˚C and rapidly cooled in an ice bath. Using a spectrophotometer, the samples were measured against a reagent blank at 700 nm. A standard curve was plotted with Trolox (12 to 400 mg L^{-1}: $R^2 = 0.995$). Sample results were expressed as mg TE/100 g powder.

Ferric reducing antioxidant power (FRAP) assays were conducted as outlined by Benzie and Strain (1996). A sample extract of 0.1 mL was added to 0.9 mL of distilled H_2O and 2

mL of FRAP reagent (composed of 1 mL of $FeCl_3 \cdot 6H_2O$ (0.02 M), and 10 mL of ammonium acetate buffer (0.3 M)). The samples were left to react for 5 minutes at ambient temperature and were spectrophotometrically measured at 593 nm against a reagent blank (2 mL FRAP reagent and 1 mL H_2O). Trolox solutions with concentrations varying from 5 to 170 mg L^{-1} were used to generate a standard curve with $R^2 = 0.9992$. Results were expressed as mg TE/100 g powder.

5.2.6 Total phenolic content (TPC) by Folin-Ciocalteu's assay

The Folin-Ciocalteu's (F-C) assay was conducted as outlined by Singleton et al. (1999) with some alterations. Sample extracts from the antioxidant analysis (section 2.5) were used, whereby 1 mL of sample or standard was transferred to a glass vial and mixed with 500 µL of F-C phenol reagent. After 5 min, 1.5 mL of 20% Na_2CO_3 was added. The mixture was vortexed for 10 seconds, covered with aluminium foil and placed in a dark room at ambient temperature for 2 hours. The solutions were then transferred into cuvettes and read at an absorbance of 756 nm against a water blank. Gallic acid solutions ranging from 0 - 200 mg/L ($R^2 = 0.999$) were used for calibration. The TPC of samples were expressed as gallic acid equivalents (mg GAE/g).

5.2.7 Extraction and analysis of amygdalin using LC-MS

An extraction method from Bolarinwa et al. (2014) was adapted with slight modifications. Samples (2.0 ± 0.1 g) were mixed with ethanol (50 mL) in a 250 mL round bottom flask. The mixture was refluxed for 100 min and then cooled down to room temperature. Whatman No.1 filter paper was used to filter the refluxed sample into another round bottom flask. A Buchi Rotavapor (R – 215) connected to a Buchi Vacuum Controller (V – 850), Buchi Recirculating Chiller (F – 100) and a temperature-controlled Buchi water bath (B – 491) were used to

remove ethanol from the extract (35 °C, 7 mbar). Diethyl ether (10 mL) was added to the dried sample and the mixture was vortexed (1 min) at room temperature (20 ± 2 °C) to precipitate amygdalin. The diethyl ether was left to evaporate overnight in the fume hood and precipitated amygdalin was dissolved in MilliQ water (5 mL) and transferred into Eppendorf tubes (1.5 mL). Eppendorf tubes were centrifuged (10 min, 22 °C, 10,000 rpm) and filtered through PTFE filters (0.45 µm) into amber vials (1.5 ml), ready for LC-MS analysis.

Quantification was performed using commercial amygdalin standards to generate a calibration curve in the range of 0.195 – 25.0 mg L (R^2 = 0.991). The LC-MS analysis was carried out using an Agilent 1260 Infinity Quaternary LC System (California, USA). The LC-MS comprised of 1260 infinity quaternary pump, 1260 infinity ALS sampler and a 1260 infinity TCC column component connected to a 6420 triple quadrupole LC/MS system with multimode ionisation source.

The Waters XSelect CSH C18 (2.1 x 100 mm, 3 µm) column was used for analysis of amygdalin. The mobile phases were composed of water containing 0.1% (v/v) formic acid (A) and acetonitrile and 0.1% (v/v) formic acid (B). The initial gradient conditions were 95:5 (A:B). From 0 to 8 min, B was increased to 90% and held for 2 min, then from 10 min to 11 min, B was decreased to 5%. The injection volume was 3 µL and the total run time for each sample was 20 mins.

The MS ionisation source conditions were set to a capillary voltage of 4 kV and corona current of 4 µA. Drying gas (N_2) temperature of 300˚C at a flow rate of 5 L/min, vaporiser temperature of 250˚C and nebuliser pressure of 20 psi was used. A positive ion mode was used with MRM for quantitative analysis. Precursor-to-product ion transition used for amygdalin was [M+H] + m/z 456 (qualifier) → 323, 221 (quantifier) with a fragmentor voltage of 220 V and collision energy of 3 eV and 8 eV, respectively.

5.2.8 Extraction and analysis of persin using NMR spectroscopy and LC-MS

The persin standard was obtained by extraction of avocado leaves as outlined by Oelrichs et al. (1995) with slight modifications. Milled freeze-dried avocado leaves were extracted with $CHCl_3$ (chloroform) for 18 hours using a Soxhlet apparatus. Excess solvent was removed using a rotary evaporator (40 °C, 450 mbar) and the crude extract was analysed by ^1H NMR. The crude sample was further purified by flash column chromatography (silica gel) using 10% EtOAc in 1% CH_2Cl_2 as the eluent, affording persin as a colourless oil. This purified sample was analysed by ^1H and ^{13}C NMR using a Bruker Ascend spectrometer operating at 400 MHz for ^1H nuclei and 101 MHz for ^{13}C nuclei. The NMR spectra can be found in *Supplementary Information S2.1*. The obtained NMR data are consistent with that previously reported in the literature (Kawagishi et al., 2001), supporting the successful isolation of persin. Samples to be analysed for persin content were extracted using the Soxhlet method, as described above. Approximately 0.1 g of the leaf extract was filtered through a plug of silica using 10% EtOAc in CH_2Cl_2 and concentrated *in vacuo*. The samples were then redissolved in ethyl acetate and diluted to 20 µg/mL using methanol.

Quantification was performed with isolated persin from avocado leaf to generate a calibration curve ranging from 0.188 – 3 µg/mL (R^2 = 0.9931) using LC-MS. The LC-MS analysis was carried out using same set up described in *section 2.7* above.

The Poroshell 120 EC-C18 (2.1 x 50mm 2.7 µm) column was used for analysis of amygdalin. The mobile phases were composed of water containing 0.1% (v/v) formic acid (A) and acetonitrile and 0.1% (v/v) formic acid (B). The initial gradient conditions were 60:40 (A: B). From 0 to 1 min, B was then increased to 65% and held for 3 min, then from 4 min to 5 min, B was increased to 90% and then reduced to 40 % from 5 to 6 minutes. The injection volume was 1 µL and the total run time for each sample was 11 min and 30 sec.

The MS ionisation source conditions were set to a capillary voltage of 4 kV and corona current of 4 µA. Drying gas (N_2) temperature of 300˚C at a flow rate of 10 L/min, vaporiser temperature of 250˚C and nebuliser pressure of 30 psi was used. A positive ion mode was used with MRM for quantitative analysis. Precursor-to-product ion transition used for amygdalin was [M+H] + m/z 381 (qualifer) → 363, 285 (quantifier) with a fragmentor voltage of 100 V and collision energy of 1 eV and 4 eV, respectively. Chromatographic repeatability (n = 10) was estimated and the residual standard deviation (RSD), limits of detection (LOD) and limit of quantification (LOQ) was calculated as outlined by Permal, Chang, et al. (2020). RSD, LOD and LOQ was 6.8%, 0.07 µg/mL and 0.25 µg/mL respectively.

5.2.9 Statistical analysis

Samples were analysed in triplicate with data expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) along with Tukey's pairwise comparison of means was performed using the XLSTAT software (version 2021.2.2). A difference of $p \leq 0.05$ was considered significant.

5.3 Results and discussion

5.3.1 Physical properties of extrudates

The expansion characteristics and physical properties of extruded snacks are crucial for acceptability by consumers as most extruded snacks are expected to have a puffed structure (Ainsworth et al., 2007). Results from **Table 1** indicated that avocado seed extrudates expanded the least, approximately 14%. Malt barley and brown rice were expanded much higher at about 65% and 118% respectively. Brown rice had the lowest bulk density (0.195

g/cm^3), followed by avocado seed (0.229 g/cm^3) and malt barley (0.375 g/cm^3). The apparent density was lowest in avocado seed (0.598 g/cm^3), followed by malt barley and brown rice (0.614 g/cm^3 and 0.796 g/cm^3 respectively). Malt barley had the lowest porosity at 40.4% followed by avocado seed at 63.8% and the highest, brown rice at 74.7%.

The bulk density and expansion ratio are both linked when describing the extent of puffing for a sample. These two parameters give an insight into the development of pores, overall expansion, and change in structure of the cooked product. Major factors that influence bulk density and expansion ratio are amylopectin content, feed moisture content, screw speed and temperature during processing. In this study, feed moisture and screw speed were kept constant during friction cooking. Therefore, the disparity in lateral expansion of avocado seeds compared to barley and brown rice (**Table 15**) could be attributed to amylopectin in the raw materials. Approximate amylopectin content for avocado seeds, barley and brown rice has been reported to be 25.5%, 41.5% and 53.8% (wet basis) respectively (Chel-Guerrero et al., 2016; Djurle, Andersson, & Andersson, 2016; González et al., 2013; Hoover, Sailaja, & Sosulski, 1996). Amylopectin expands more than amylose because the linear chains of amylose align themselves on the shear field, making them difficult to pull apart during expansion inside the extruder barrel (Chadha et al., 2021). Evidently, as amylopectin is most abundant in brown rice, lateral expansion of brown rice was also the highest (**Figure 21**). The difference in bulk density for avocado seeds compared to barley and brown rice could be due to the high lipid content in avocado seeds. Mościcki and Wójtowicz (2011) reported that raw materials such as cereal flours with 0.5-1% oil can result in a reduction of energy and consequently temperature of the flour mix, therefore, accelerating mass flow in the die opening.

Table 15. Physical and textural characteristics of extruded avocado seed, brown rice, and malt barley.

Extrudate	Lateral expansion (%)	Bulk density (g/cm^3)	Apparent density (g/cm^3)	Porosity (%)	Hardness (N/cm^2)	Crispiness (N/mm)	Modulus of Rupture (MPa)	Flexural Modulus (MPa)
Avocado seed	14 ± 6^{c}	0.229 ± 0.020^{b}	0.598 ± 0.002^{b}	63.8 ± 1.3^{b}	4.34 ± 0.9^{b}	1.4 ± 0.3^{b}	0.75 ± 0.18^{b}	62 ± 16^{a}
Brown rice	118 ± 6^{a}	0.195 ± 0.016^{c}	0.796 ± 0.015^{a}	74.7 ± 2.2^{a}	12.2 ± 1.9^{a}	8.7 ± 1.9^{a}	1.13 ± 0.18^{a}	34 ± 10^{b}
Malt barley	65 ± 7^{b}	0.375 ± 0.042^{a}	0.614 ± 0.022^{b}	40.4 ± 3.8^{c}	10.7 ± 1.7^{a}	7.9 ± 1.7^{a}	1.32 ± 0.33^{a}	94 ± 36^{a}

Results expressed as mean ± standard deviation. Sample analysis was performed as triplicates (n = 3). Subscript letters within the columns do not differ statistically by Tukey's HSD test ($p < 0.05$).

Less shearing lowers degradation of starch granules, ultimately causing an increase in the viscosity of the plasticised mass. The increased viscosity affects the expansion ratio of extrudates and if oil content is more than 3%, it may result in compacted and dense extrudate. Raw brown rice and malted barley have a total lipid content of approximately 2.8% and 1.78% (dry basis w/w) respectively (Bravi, Marconi, Perretti, & Fantozzi, 2012; F. Wu et al., 2013). Whereas Permal, Leong Chang, et al. (2020) reported 3.7 % (dry basis w/w) for total lipid content of avocado seeds. The higher bulk density and porosity of barley extrudate compared to avocado seeds appeared counter-intuitive when taking into consideration that the lateral expansion for barley was much higher than avocado seeds (**Table 15**). The reason for disparity is that lateral expansion considers expansion in the direction perpendicular to the extrudate flow, while bulk density reflects expansion in all directions (Falcone & Phillips, 1988). Therefore, the high porosity in avocado seed extrudate was attributed to longitudinal expansion along the axis of extrudate flow as opposed to lateral expansion.

5.3.2 Textural analysis of extrudates

Avocado seeds were significantly lower in hardness (4.34 N/cm^2, $p < 0.05$) compared to brown rice and barley (12.2 N/cm^2 and 10.7 N/cm^2 respectively). The avocado seed extrudate was also lower in crispiness (1.4 N/mm, $p < 0.05$) compared to brown rice and malt barley 8.7 N/mm and 7.9 N/mm respectively). For both hardness and crispiness, brown rice and malt barley were not statistically different from each other ($p > 0.05$).

Kumar, Xavier, Lekshmi, Balange, and Gudipati (2018) suggested that the hardness of extrudates correlates with fiber content. The ability of fiber to bind to water more strongly than starch inhibits water loss at the die and hence reduces the ability for extrudate expansion. The current study found a significant decrease in hardness for avocado seed extrudate, which is composed of approximately 19.5% (w/w) fiber (Permal, Leong Chang, et al., 2020). Brown

rice and barley on the other hand contain around 2.86 % and 20.2% (w/w) total dietary fiber respectively (Djurle et al., 2016; F. Wu et al., 2013). Therefore, fiber did not dictate the hardness of extrudates in this study. Instead, the level of hardness may be an effect of lateral expansion (%) – avocado seeds had the lowest level of lateral expansion compared to the other two grains.

Determination of mechanical strength and stiffness of extrudates involved the measurement of intensive properties such as Flexural Modulus (FM) and Modulus of Rupture (MoR). MoR represents the maximum bending stress of a material before breakage and FM represents the resistance to bending in a material (Callister & Rethwisch, 2014). **Table 15** showed that MoR followed similar patterns to hardness, with avocado seed extrudate having significantly ($p < 0.05$) lower rupture pressure of 0.75 MPa compared to brown rice and barley (1.13 MPa and 1.32 MPa respectively). No statistical difference ($p > 0.05$) in MoR was observed between brown rice and barley. Interestingly FM of the samples showed a different pattern to crispiness. Brown rice extrudate had the lowest FM (34 Pa), followed by avocado seed (62 Pa) and barley (94 Pa). Both avocado seed and barley had significantly higher ($p < 0.05$) FM than rice but were not significantly different ($p > 0.05$) to each other. However, low MoR of avocado seed extrudate indicates that it will break at a relatively low force, making avocado seed extrudate more brittle compared to brown rice and barley extrudates. This is also reflected in the low crispiness of avocado seed (1.4 ± 0.3 N/mm). The higher crisp value in rice extrudate is most likely due to a larger lateral % expansion, increasing the level of rigidity.

Figure 16. Brown rice (A), malt barley (B) and avocado seed (C) extrudates produced from the friction cooker Zapmill™ under the following conditions: Feed flow rate 12.8 kg/h (brown rice), 13.1 kg/h (barley) and 5.3 kg/h (avocado seed); Screw speed 50 Hz; 5 mm die opening.

5.3.3 Antioxidant capacity and total phenolics

Table 16 shows that freeze-dried avocado seeds retained the highest concentration of all antioxidant and total phenolics. This is because freeze-drying had the lowest impact on nutritional degradation compared to other drying methods (Permal, Leong Chang, et al., 2020). Alternatively, the lowest antioxidant values were observed for extruded brown rice.

There was a significant decrease in antioxidant capacity across FRAP, CUPRAC, phosphomolybdenum assays, and TPC ($p < 0.05$) in the order of freeze-dried, oven-dried and extruded avocado seed. With the FRAP assay as example, the freeze-dried avocado seed initially showed an antioxidant capacity of 56.6 mg TE (Trolox equivalent)/g. After oven-drying, this value was reduced from 22 mg TE/g to 15.4 mg TE/g when extruded, resulting in a total of 73% reduction. Avocado seed extrudate had antioxidant capacity values that were 3.8 and 17 times higher ($p < 0.05$) than barley and rice extrudate (4.04 mg TE/g and 0.90 mg TE/g) respectively. In contrast, due to a high degree of variability in the phosphomolybdenum assay, no significant differences were found between oven-dried and extruded avocado seed ($p > 0.05$)

FRAP results on antioxidant capacity of freeze-dried avocado seed as shown in **Table 16** was comparable to results reported by Ortega-Arellano, Jimenez-Del-Rio, and Velez-Pardo (2019), but lower than results reported by Permal, Leong Chang, et al. (2020). Furthermore, the total phenolics found in raw seed was lower compared to results reported by W. Wang et al. (2010). The decrease in antioxidant activity of extruded snacks was also reported by Nayak, Berrios, Powers, and Tang (2011). The authors observed a significant decrease ($p < 0.05$) in total antioxidant capacity (TAC) and total phenolics for raw purple potato and yellow pea flour formulations (35:65 and 65:35, w/w) after extrusion at 140˚C. The authors explained that this decrease in TAC may have been a result of phenolic compounds binding to the protein matrix of formulated flours. Another explanation for the decrease in antioxidant activity, and TPC could be due to phenolic decarboxylation. High extrusion temperatures and moisture content may promote the polymerisation of phenols and tannins leading to reduced extractability and antioxidant activity (Morales et al., 2015).

Antioxidants play an important role in protecting against diseases by reacting with oxidative free radicals, chelating transition metals, reducing peroxides and stimulating anti-

oxidative defence enzyme activities (Nayak et al., 2011). However, many extruded products require fortification with vitamins and minerals to boost their nutritional value. As evident in **Table 16**, flours and grains extruded as a single ingredient are low in nutraceutical content (Camire, 2001). Yet, little fortification is required for avocado seeds as they are already high in antioxidant capacity and phenolic content. Moyer, Hummer, Finn, Frei, and Wrolstad (2002) reported FRAP values for 30 blueberry cultivars from the genus *Vaccinium*. The authors showed that the average antioxidant capacity of all these cultivars were 18.5 mg TE/g (fresh weight) which is only slightly higher than extruded avocado seed (15.4 mg TE/g).

Although the avocado seed is nutrient dense due to its high phenolic content, palatability of these extruded snacks may be an issue. Research by Figueroa, Borrás-Linares, Lozano-Sánchez, and Segura-Carretero (2018) determined the phenolic profile of avocado seed using accelerated solvent extraction (ASE) and liquid chromatography coupled to Ultra-High-Definition Accurate-Mass Q-TOF. The major phenolic compounds identified in avocado seeds were tannins, hydroxybenzoic acids, hydroxycinnamic acids and catechins which are known to impart a bitter taste. There are two possible ways to remove the bitterness in the avocado seed snack. Firstly, one can introduce a pre-treatment step of cooking the avocado seeds before processing into a RTE snack. Secondly, the introduction of sugar or honey can mask the bitterness in the snack. However, future organoleptic analysis should focus on developing the palatability of the extruded avocado seeds.

Table 16. Antioxidant capacity and total phenolic content of avocado seed, malt barley and brown rice

Sample	Antioxidant capacity – Trolox equivalents (mg/g)			Total phenolics – Gallic acid equivalents (mg/g)
	FRAP	CUPRAC	Phosphomolybdenum	FC
Freeze-dried avocado seeds	57.6 ± 4.1 [a]	186.1 ± 2.8 [a]	313 ± 26 [a]	31.2 ± 2.9 [a]
Oven-dried avocado seeds	22.7 ± 1.2 [b]	58.0 ± 1.9 [b]	140.6 ± 1.2 [b]	16.9 ± 0.5 [b]
Extruded avocado seeds	15.4 ± 1.8 [c]	39.2 ± 0.4 [c]	105 ± 12 [b]	12.9 ± 1.1 [c]
Extruded barley	4.04 ± 0.2 [d]	10.1 ± 1.2 [d]	52.6 ± 9.7 [c]	3.4 ± 0.2 [d]
Extruded brown rice	0.90 ± 0.2 [e]	4.3 ± 1.0 [e]	21 ± 14 [c]	0.7 ± 0.1 [e]

Results expressed as mean ± standard deviation in dry weight basis. Same superscript letters indicate no statistically significant difference between rows as indicated by ANOVA and Tukey's HSD test. Samples analysis was performed in triplicates (n = 3)

Although the avocado seed is nutrient dense due to its high phenolic content, palatability of these extruded snacks may be an issue. Research by Figueroa et al. (2018) determined the phenolic profile of avocado seed using accelerated solvent extraction (ASE) and liquid chromatography coupled to Ultra-High-Definition Accurate-Mass Q-TOF. The major phenolic compounds identified in avocado seeds were tannins, hydroxybenzoic acids, hydroxycinnamic acids and catechins which are known to impart a bitter taste. Therefore, future work should focus on the organoleptic properties of the extruded avocado seeds.

5.3.4 Amygdalin content

LC-MS analysis on freeze-dried, oven-dried, and extruded avocado seeds only contained trace amounts of amygdalin (7.5×10^{-7} mg/g, 7.2×10^{-7} mg/g and 2.6×10^{-6} mg/g, respectively) that were all below the lowest amygdalin standard concentration (1.95×10^{-4} mg/g). Dang, Nguyen, and Tran (2017) reported that 500 mg of amygdalin contains around 30 mg of cyanide. In the case of avocado seed extrudate, the concentration of cyanide would be 1.56×10^{-7} mg/g, which can be deemed insignificant as acute cyanide toxicity occurs in humans only at doses between 0.5 and 3.5 mg/kg body weight (Bolarinwa et al., 2014). Alternatively, amygdalin concentration in apricot kernel (positive control) was found to be 53.8 ± 7.4 mg/g. Zhang, Zhang, Yao, and Zhang (2019) reported Similar amygdalin concentrations of 64.68 mg/g of apricot seed. Hence the extraction and quantification methods carried out in this study are valid, and confirmed that amygdalin was not present in freeze-dried avocado seeds, at a detection limit of 1.95×10^{-4} mg/g. As far as amygdalin concentration is concerned, avocado seeds and its extrudate were found to be not cyanogenically toxic for human consumption. It is known that the presence of amygdalin in foods can impart a bitter taste (Bolarinwa et al., 2014). However, as the concentration of

amygdalin is low in the extruded avocado seeds, any bitter or unpleasant taste in the snacks might be attributed to its high phenolic content.

5.3.5 Persin content

The persin standard used for determination of persin concentration was obtained from avocado leaves using a modified procedure described by Oelrichs et al. (1995). Following purification by flash column chromatography, analysis by ^{1}H NMR, ^{13}C NMR and mass spectrometry showed that the characterisation data matched those previously reported. Prior to purification by chromatography, analysis by ^{1}H NMR showed that the crude extracts, from avocado leaves, contained a large proportion of persin, as evidenced by distinct and strong signals at 2.43 ppm and 2.61 ppm, amongst others. The high integration of signals in the range of 5.28-5.42 ppm also indicated the presence of a high concentration of oleic acid in the crude avocado leaf extract. Kawagishi and co-workers (2001) also reported the presence of four other fatty acid derivates of persin in avocado, as shown in **Figure 23** (compounds **3**-**6**). Compounds **3**-**6** each contain alkenes conjugated with ketones that give characteristic signals from 6-7 ppm in the ^{1}H NMR. However, the absence of any signals in this region in the ^{1}H NMR of the crude leaf extract suggest that these compounds were not present in avocado leaves but were present in extracts of the avocado seed. During flash chromatography, it was found that persin degraded readily if left for long periods of time on the silica medium. The best results for obtaining a high yield of persin required the use of a quick flash column with silica using 10% EtOAc in CH_2Cl_2 as the eluent.

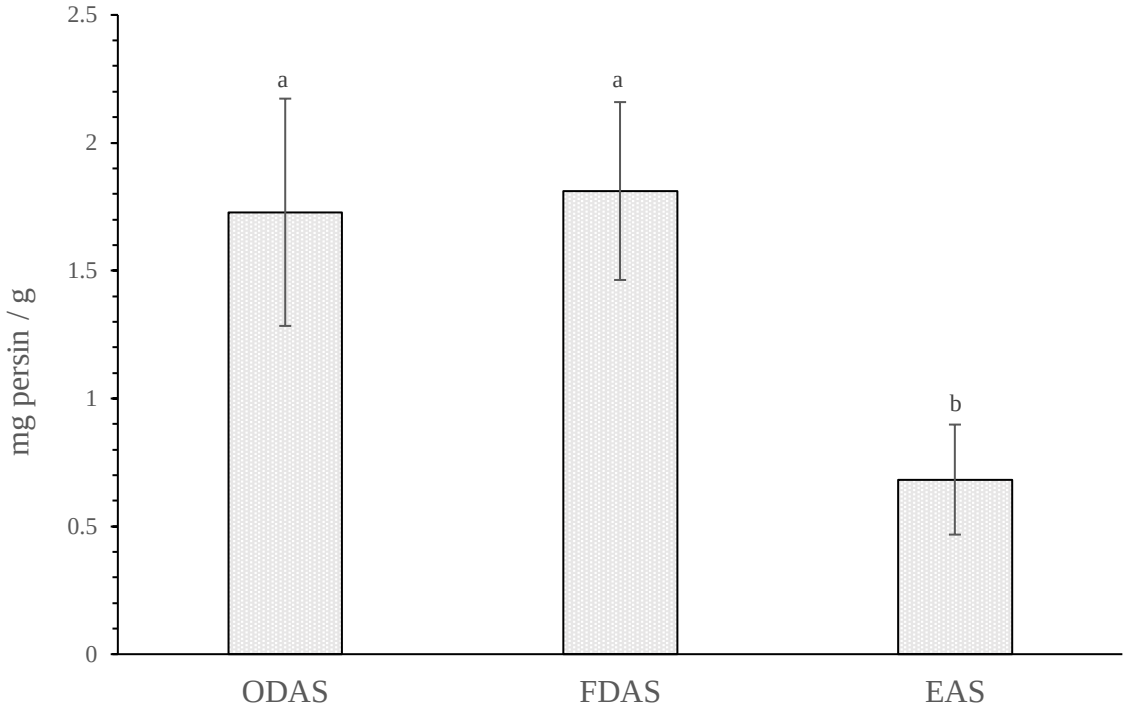


Figure 17. Persin concentration in oven dried (70˚C, 60 hours) avocado seed (ODAS), freeze-dried avocado seed (FDAS) and extruded avocado seed (EAS), based on dry weight (DW). Each data point was measured in triplicates (n = 3), where error bars represent standard deviation of means. Superscript letters that are not the same do not differ statistically based on Tukey's test ($p < 0.05$).

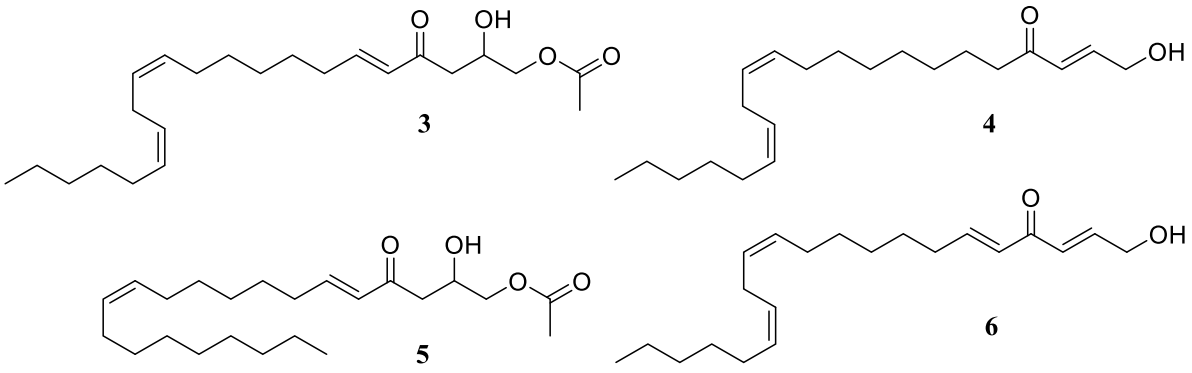


Figure 18. Persin and persin analogues as reported by Kawagishi et al. (2001).

Persin concentration in oven-dried and freeze-dried avocado seeds (**Figure 22)** were not significantly ($p > 0.05$) different to each other (1.73 ± 0.44 mg persin/g and 1.81 ± 0.35 mg persin/g respectively, based on DW). However, oven-dried, and freeze-dried avocado seeds were significantly ($p > 0.05$) higher in persin, compared to extruded avocado seed (0.68 ± 0.22 mg persin/g). Thermostability of persin was assessed. Results showed that isolated persin was stable at temperatures up to 130˚C (*Supplementary information S2.3).* Therefore, the degradation of persin from friction cooked avocado seed was most likely a result of the persin reacting with other compounds in the mixture during extrusion. Rodríguez-López, Hernández-Brenes, and Díaz de la Garza (2015) found that persin concentration in fresh avocado flesh ranged from 0.05 – 1.3 mg/g of fresh weight (FW) (0.018 – 0.47 mg/g, DW). The result from this study shows that persin concentration in extruded avocado seeds was only slightly higher than in the avocado flesh at dry weight.

A study by Craigmill, Seawright, Mattila, and Frost (1989) investigated the effects of persin that involved orally dosing freeze-dried avocado leaves (20 g/kg) to goats. Results showed that goats suffered a loss in milk production, due to a specific necrosis of the secretory epithelium of the mammary gland. A similar result was also observed in lactating mice fed with freeze-dried avocado leaves (Sani, Seawright, Ng, O'Brien, & Oelrichs, 1994), which led to the isolation of the active persin agent [(+)-(*Z*,*Z*)-1-(acetyloxy)-2-hydroxy-12,15-heneicosadien-4-one. Enantioselective syntheses of *S* and *R* enantiomers of persin showed that only the *R* enantiomer was involved in the injury to lactating mammary gland dosed in the range of 60–100 mg/kg. Necrosis of the myocardial fibres and hydrothorax were reported for doses over 100 mg/kg, and a fatal dose at 200 mg/kg (Oelrichs et al., 1995). On the other hand, Butt et al. (2006) demonstrated the positive effects of persin and its potential to combat human breast cancer cells. However, these studies were conducted on mice and clinical trials that have yet to investigate the effect of persin in humans. Regardless, avocado

flesh is consumed regularly and contain persin concentrations of 0.018–0.47 mg/g DW. Hence in terms of extruded avocado seeds, this product should be considered safe to consume due to its low persin concentration. On the other hand, consumption of raw freeze-dried avocado seed is not recommended as the concentration of persin is almost 2.6 times higher than the extruded avocado seeds.

5.4 Conclusion

The present work aimed to investigate the potential utilisation of waste avocado seeds to produce a crispy snack product. Findings in this study showed that avocado seeds could be successfully extruded into a porous puffed consumable snack. The texture of the extrudate was found to be less crispy, hard and more brittle compared to rice and barley extrudates. The high antioxidant capacity of avocado seeds decreased by 58% when oven-dried and 69% when extruded. Yet, despite the substantial decrease, the antioxidant capacity of the extruded seeds was still comparable to antioxidant-rich blueberries. With regards to the safety of the extruded snack, no amygdalin was detected in the freeze-dried avocado seed and extruded avocado seed snack. The effects of persin on the human body has not yet been established. However, the concentration of persin in the avocado RTE snack from this study was 0.68 mg/g (DW) that was only slightly higher than avocado flesh (0.018 – 0.47 mg/g). Therefore, the health implications of amygdalin and persin in the avocado RTE snack were not of concern. Overall, the extrusion process of avocados shows promise in valorising the seed waste into a snack product without entirely losing its antioxidant properties as a functional food. Future work to determine the organoleptic properties of the RTE avocado seed snack is recommended to determine its acceptability

6 Chapter 6: Overall Conclusion

6.1 Thesis background

As the demand for avocado oil rises due to popularity, an abundance of by-products produced increases proportionately. However, with the research techniques discussed in this thesis, we have only touched the surface on various ways that these by-products can be valorised or upcycled into something of higher value rather than discarding them into landfill. The following section summarises each of the research chapters and potential industrial applications of CPAO by-products and how they may be converted into a useful product.

6.2 Research Summary

6.2.1 Chapter 3 summary

The production of commercial cold pressed avocado oil (CPAO) generates large quantity of organic wastes such as pomace, seeds, peels and wastewater. During the early harvest season, for every 1000 kg of avocado fruits processed, roughly 80 kg of oil is produced, and wastewater accounted for the highest proportion (500 kg). Therefore, it is important to find an alternative application for this wastewater rather than its direct disposal into landfills. Proximate analysis, total phenolic content (TPC) and antioxidant assays were conducted on the avocado wastes. Avocado wastewater (AWW) was spray dried into powder at different temperatures from 110 °C to 160 °C, which concomitantly increased the TPC and antioxidant capacities of the AWW powder. The powder was further applied as a preservative in pork sausages and was found to be effective in preventing lipid oxidation.

6.2.2 Chapter 4 summary

Avocado wastewater (AWW) is the largest by-product of cold pressed avocado oil. The aim of this study was to valorise AWW by converting it into spray dried powder for use as a lipid peroxidation inhibiting food preservative. To increase the powder yield of AWW, addition of carriers and spray drying parameters (temperature and feed flow rate) were optimised. The highest AWW powder yield was 49%, and was obtained using 5% whey protein concentrate (WPC), with a feed flow rate of 5.8 g/min and an inlet drying temperature of 160 ◦ C. The liquid chromatography mass spectrophotometry (LC-MS) analysis showed that AWW encapsulated with WPC had the highest retention of α-tocopherol (181.6 mg/kg powder). AWW with 5% WPC was tested as a preservative in pork fat cooked at 180 ◦ C for 15 min. Thiobarbaturic acid reactive substances (TBARS) assay showed that the effectiveness of AWW powder was comparable to commercial additives such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and sodium erythorbate (E316).

6.2.3 Chapter 5 summary

Avocado seeds account for 13% of the waste from industrial production of cold pressed avocado oil (CPAO). Therefore, the aim of this study was to valorise avocado seeds by converting it into an extruded snack product using a friction cooker and comparing their textural and physical characteristics to extruded brown rice and malted barley ready to eat (RTE) snacks. Concentration of toxins; amygdalin and persin were compared in extruded avocado seed and fresh avocado seeds. Avocado seed extrudates were significantly lower in lateral expansion, apparent density, porosity, hardness, and crispiness compared to brown rice extrudates. Antioxidant capacity and total phenolic content (TPC) was highest in freeze-dried avocado seeds. Antioxidant capacity and TPC of avocado seed extrudates were significantly higher than brown rice and malted barley. The concentrations of both amygdalin and persin

in the RTE avocado seed snack were present at non-toxic levels (2.6 ×10 -6 mg/g and 0.68 mg/g respectively).

7 Supplementary information

7.1 S.1.1 Analysing for colour in avocado powder

The avocado powder was tested for colour using a Nix™ Pro Color Sensor (Nix Sensor Ltd., 14 mm aperture size). Approximately 4 g of samples were filled into zip lock polyethylene bags and scanned using the sensor. Results were collected via smartphone pairing showing CIE values. L^* represents darkness to lightness on a scale of 0-100, a^* is a coordinate (-120 to 120), representing the scale of greenness through to redness, and b^* represents negative values for blueness and positive values for yellowness. Readings were replicated 3 times on each independent batch.

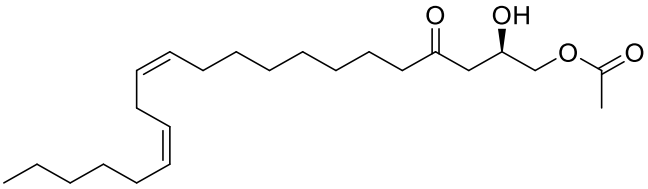
7.2 S1.2 Discussion on avocado powder colour

Colour is a very important feature for visual acceptance by consumers. Colour measurements are presented in Table 2. For powdered avocado black water, the a* value was 0.2. This was expected as avocado pulp is naturally green, so the resulting avocado water is closer to the colour of its flesh. Greenness changed significantly with increased inlet temperature. Increasing the inlet temperature from 110˚C to 160˚C, increased the a* value from 0.2 to 2, shifting away from greenness and increasing in redness. Similarly, b* increased from 25 to 30.9 ($p < 0.01$), from a blue tone towards yellow. However, L* decreased from 69.4 to 64 ($p > 0.05$) indicating production of a darker powder with increased inlet temperature. With the significant increase in red (a*) and yellow (b*) values, it is evident that increasing inlet temperature oxidises or caramelises the sample due to pigment oxidation and thermal exposure. Although lightness slightly decreased with increased inlet temperature, there was no statistical significance to define why this was the case. Mishra, Mishra, and Mahanta (2014) and Araújo, Rodriguez-Jasso, Ruiz, Pintado, and Aguilar (2018) reported a

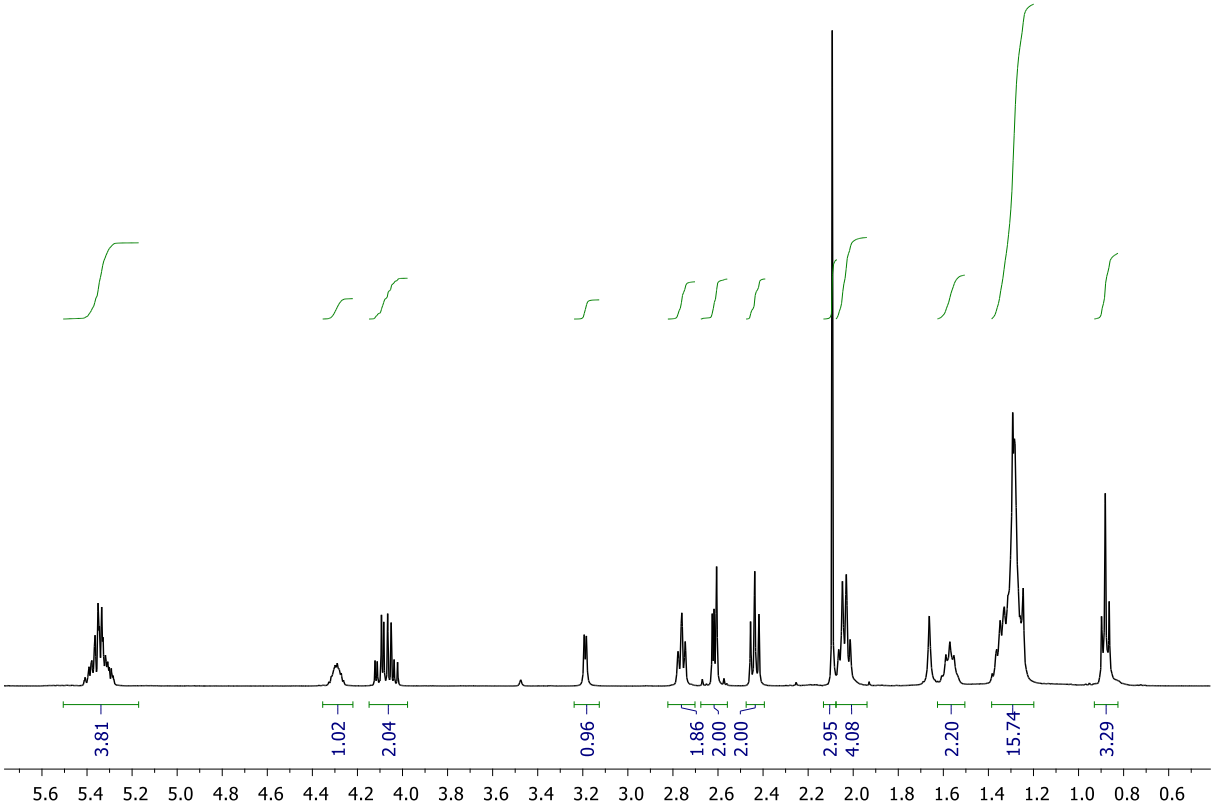
significant increase in L* value as a result of maltodextrin concentration. As maltodextrin is white, a greater percentage of this material made samples lighter, consequently increasing L* values higher. Samples in this study did not contain any carrier components. Therefore, it was expected that the lightness would have decreased.

7.3 S2.1 ^{1}H and ^{13}C NMR spectra

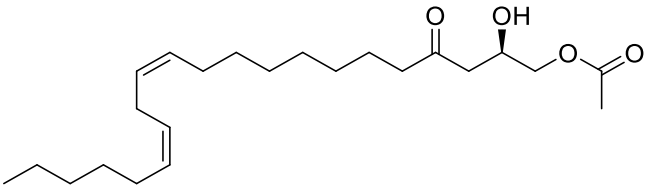
(2*R*,12*Z*,15*Z*)-2-Hydroxy-4-oxohenicosa-12,15-dien-1-yl acetate (persin)



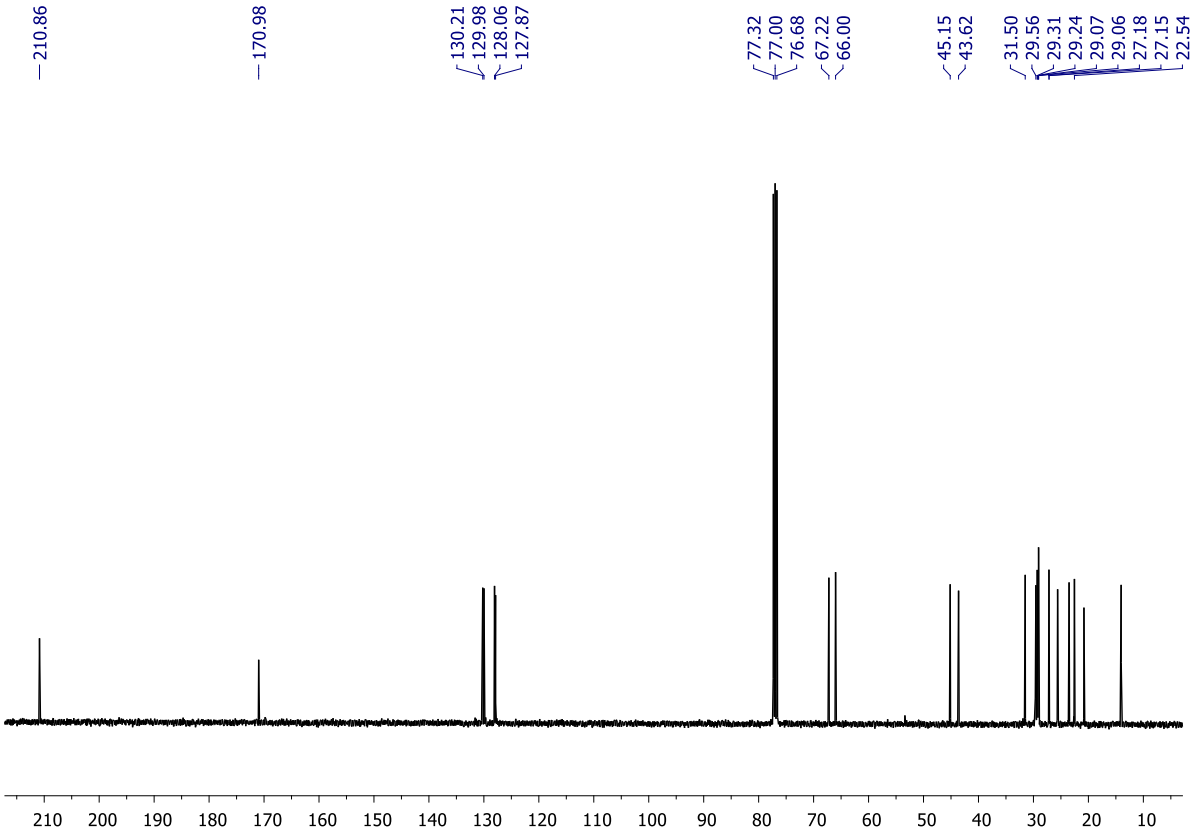
^{1}H NMR (400 MHz, $CDCl_3$):



(2*R*,12*Z*,15*Z*)-2-Hydroxy-4-oxohenicosa-12,15-dien-1-yl acetate (Persin)

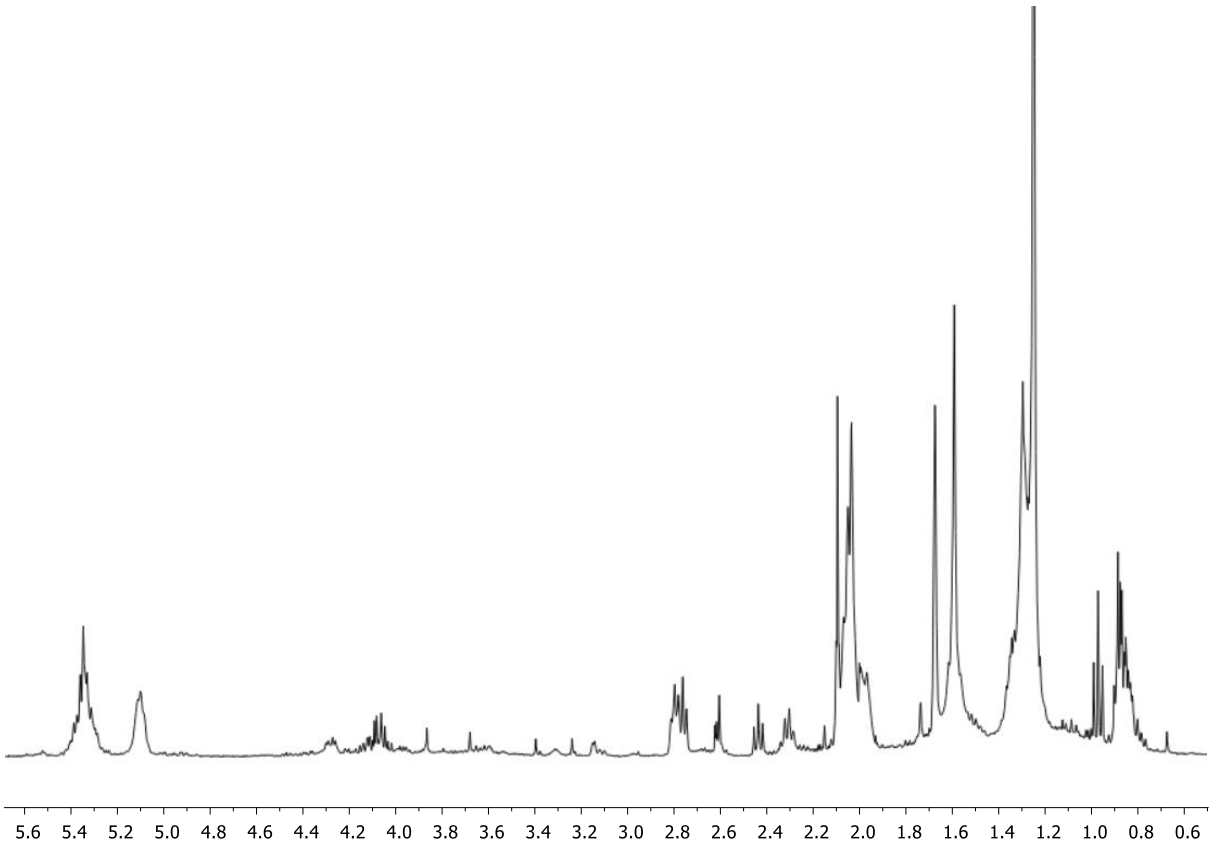


^{13}C NMR (101 MHz, $CDCl_3$):



^{1}H NMR spectrum of the crude extract from avocado leaves

^{1}H NMR (400 MHz, $CDCl_3$):



7.4 S2.2 Zapmill™ Friction Cooker

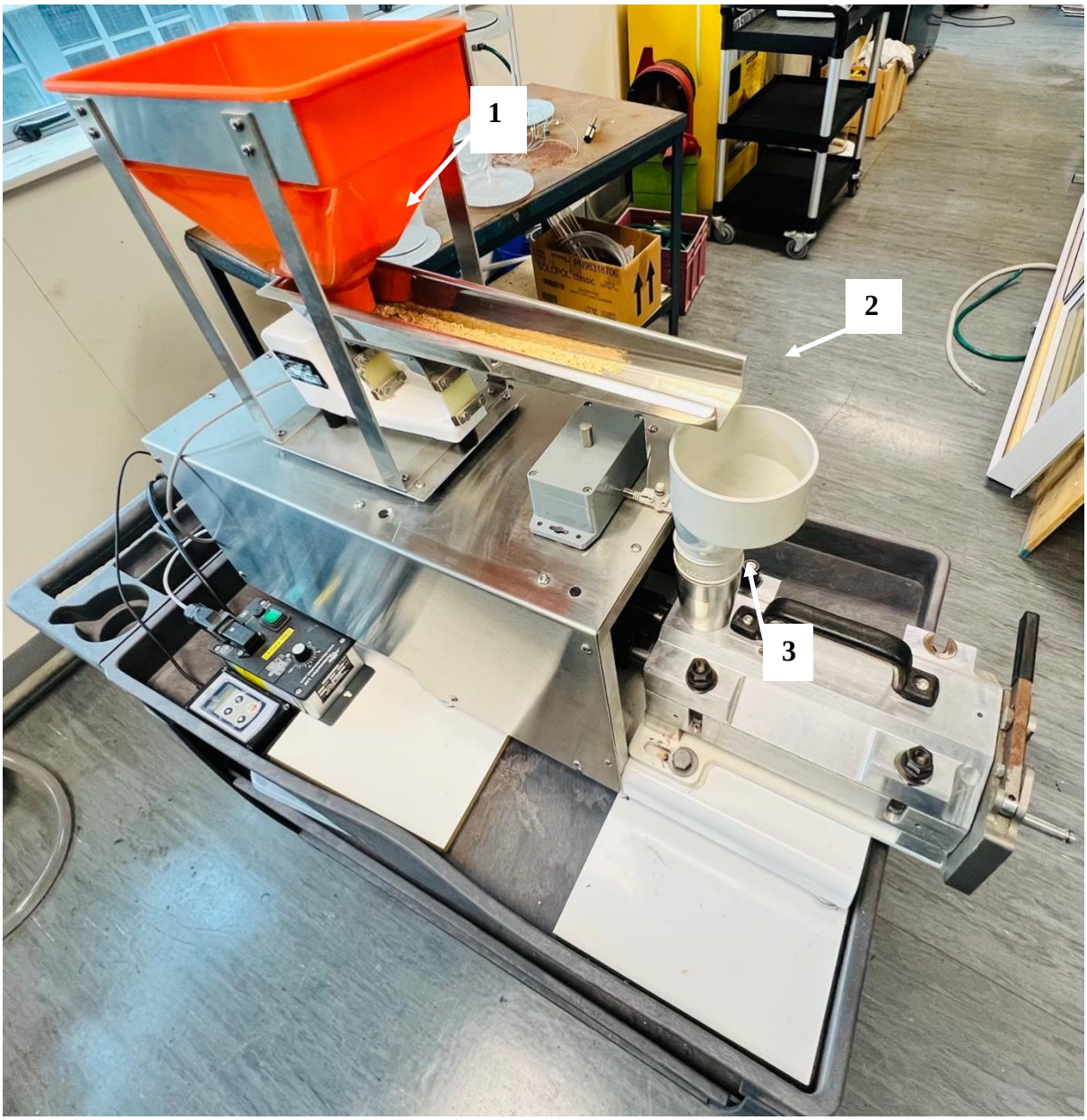


Figure S-19. Full setup of the Zapmill™ Friction Cooker. Vibration feeder (1), extrusion hopper (2) and extrusion chamber (3).

Figure S-20. Extrusion chamber enclosure with exposed screw shaft (1). The screw shaft rotation speed was maintained at 50 Hz during extrusion.

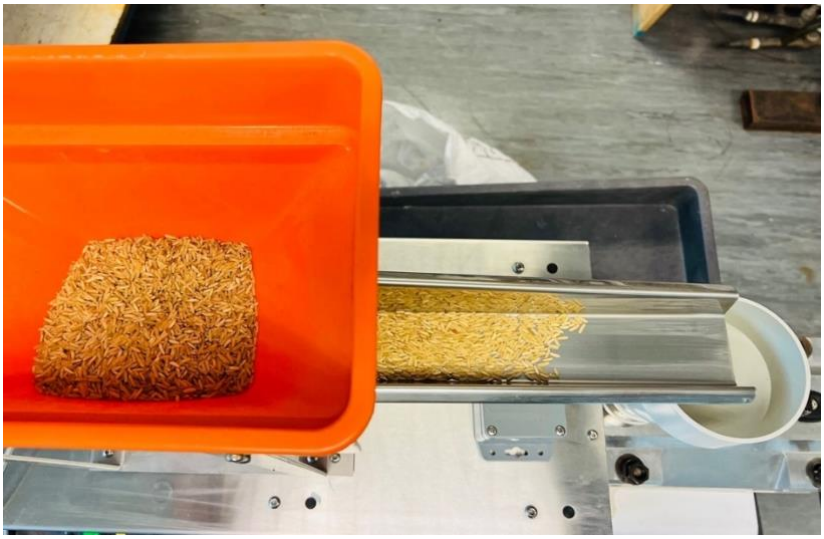


Figure S-21. Vibration feeder filled with brown rice, that will feed down the extrusion hopper and into the extrusion chamber. Feed flow rate was fixed at 5.3, 13.1 and 12.8 kg/h for avocado seeds, brown rice, and barley respectively.

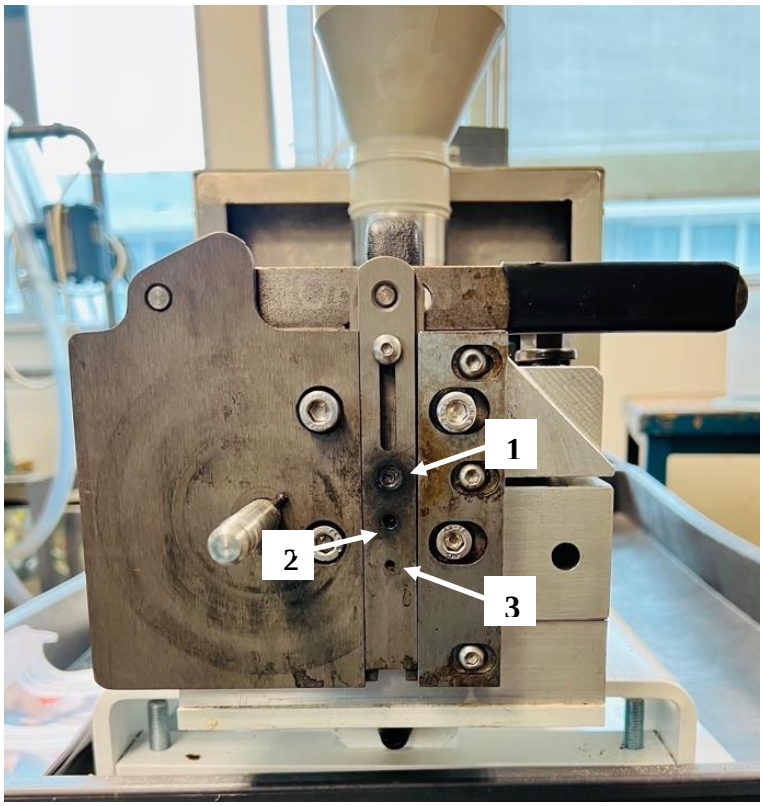


Figure S-22. Die sizes 8 mm (1), 5 mm (2) and 3 mm (3). The feed material is cooked as a result of friction created by the rotating screw shaft while exiting the die.

Figure S-23. Control box for vibration feeder (1) and control panel for screw shaft (2).

7.5 S2.3 Persin thermostability

A sample of persin (15 mg) was first dissolved in deuterated chloroform and analysed by a Bruker 400 MHz NMR spectrophotometer. This sample was then transferred into a 100 mL round bottom flask and the solvent removed under low pressure. The sample of persin was then placed in an oven at 130˚C for 5 minutes. This persin sample was redissolved in deuterated chloroform and analysed again using the ^{1}H and ^{13}C NMR.

Results from the NMR spectra indicated that the isolated persin from avocado leaf showed no changes in its NMR spectra. This indicated that the isolated persin was stable when subjected to 130˚C heat. Therefore, the decrease of persin observed in the RTE avocado seed snack, compared to ODAS and FDAS was most likely a result of the persin reacting with other compounds in the avocado seed mixture during the friction cooking process.

7.6 Testing for antifungal properties in avocado wastewater (AWW)

7.6.1 Introduction

To sustain a growing population, there is not only a need to produce more food but to assure that the food being produced is safe for human consumption. One of the most common factors contributing to hazardous foods is microbial contamination (Chia & Dykes, 2010). There have been several synthetic additives introduced by the food industry to control food spoilage by inhibiting growth of microbial pathogens. Yet, in the last decade, western societies appear to be experiencing a trend towards "green" consumerism, favouring less synthetic food additives or products that have a smaller impact on the environment (Conte, Speranza, Sinigaglia, & Del Nobile, 2007). Traditionally, herbs and spices have been utilised to either enhance aroma or increase shelf life of packaged foods. Moreover, natural compounds from edible plants such as oilseeds, spices, fruits, and vegetables have been extensively researched as food preservatives and possible replacements for chemical additives (Conte et al., 2007; Permal, Chang, et al., 2020; Permal, Leong Chang, et al., 2020).

Plants possess antimicrobial activity that can inhibit the growth of food pathogens and spoilage microorganisms. Plant materials such as grape (*Vitis vinifera L.*) seeds and *Citrus spp.* peel (Baydar, Sagdic, Ozkan, & Cetin, 2006; Johann et al., 2007) are natural products that display antimicrobial activity and have been applied to foods. Extract from the skin of immature avocado fruits (*Persea Americana Mill.)* has demonstrated both antifungal and antibacterial properties (Jacob & Young, 1971; Sivanathan & Adikaram, 1989). Moreover the seeds of immature avocado fruits have also been found to contain antibacterial properties (Jacob & Young, 1971). Antifungal properties of immature avocado were discovered in the idioblast oil cells, which are only found in the avocado leaves, seeds, roots and mesocarp of

the avocado fruit (Cummings & Schroeder, 1942; Platt & Thomson, 1992; Platt-Aloia, Oross, & Thomson, 1983). Idioblast cells contain alkaloids, sesquiterpene hydroperoxides (Platt & Thomson, 1992), a group of 2-alkylfurans and persin. These compounds can inhibit the growth of fungal pathogens such as, *Colletotrichum gloeosporioides* and *Colletotrichum acutatum* (Adikaram, Ewing, Karunaratne, & Wijeratne, 1992; Hartill, 1991; Rodriguez-Saona, Millar, Maynard, & Trumble, 1998; Rodriguez-Saona & Trumble, 2000). Catechin flavones, tannins and polyphenolic compounds are often found in the flesh and seed avocado fruit. These compounds are all antimicrobial in nature and can contribute towards the antibacterial activity in immature avocado fruits. Inhibition of microbial growth from avocado flesh and seed extracts were tested against *Staphylococcus aureus*, *Salmonella* Enteritidis, *Citrobacter freundii*, *Pseudomonas aeruginosa* and *Enterobacter aerogenes* (Chia & Dykes, 2010; Jacob & Young, 1971; J. J. G. Leite et al., 2009). Chia and Dykes (2010) also found that even after maturity, the avocado skin and seed still possessed antifungal and antibacterial activities.

7.6.2 Antifungal properties in avocado skin

Hartill (1991) and Adikaram et al. (1992) described that avocado anthracnose, caused by *Collectotrichum gloeosiporioides* is a fungal disease contributing to post harvest rotting in avocado fruit that is not seen in unripe avocados. However, the decaying process develops rapidly during ripening, which indicate the presence of latent infection. This delayed onset of infection has been attributed to the presence of significant concentration of antifungal compounds in the skin of avocado fruit during early stages of growth (Prusky, Keen, Sims, & Midland, 1982). Bowen et al. (2018) reported a range of antifungal compounds that have been identified from avocado skin, with the most active fungal compound being (*Z*,*Z*)-1-acetoxy-2-hydroxy-4-oxoheneicosa-12, 15-diene (Adikaram, Egodawela, & Karunaratne,

1993; Prusky et al., 1982). This compound is more commonly known as persin. Interestingly, persin supresses vegetative growth of *C. gloeosporioides* during ripening and is rapidly lost during ripening of the avocado fruit. It has been suggested that the decrease in persin during ripening could be a result of lipoxygenase activity (Bowen et al., 2018).

Avocado contains secondary metabolites of flavonoids in the form of catechin. Research by Karni, Prusky, Kobiler, Bar-Shira, and Kobiler (1989) indicated that lipoxygenase activity is mediated by epicatechin which inhibits avocado lipoxygenase. Epicatechin concentration in avocado skin declines during ripening, reducing the inhibitory effect on lipoxygenase, and therefore resulting in the breakdown of persin. To put this into perspective, Prusky and Keen (1993) reported that the concentration of epicatechin in avocado cv. Feurte fruit peel decreased from 500 $\mu g.g^{-1}$ fresh weight of unripe peel down to approximately 8 $\mu g.g^{-1}$ fresh weight in ripe fruits that then showed symptoms of decay from the vegetative growth of *C. gloeosporioides.* Much of the persin research has been conducted on the cultivar 'Feurte'. Prusky et al. (1982) reported that the peel of 'Feurte' and 'Hass' cultivars contained 925 mg kg^{-1} and 594 mg kg^{-1} of persin respectively (calculated on fresh weight basis). Persin content can range from 203 mg kg^{-1} for 'Nabal' and 4412 mg kg^{-1} for 'McArthur'. X. Wang et al. (2006) found that with ripening in the 'Feurte' cultivar, a decrease in persin was observed from 1820 $\mu g.g^{-1}$ in early harvested fruit to 950 $\mu g.g^{-1}$ in the late harvested fruit. This demonstrated that persin concentration in the fruit skin may change with fruit maturation.

7.6.3 Antifungal properties in idioblast cells

Idioblast cells make up for approximately 2% of the avocado flesh (Cummings & Schroeder, 1942). Research by Yang et al. (2018) utilising the light microscopy imaging technique has shown the presence of idioblast cells and oil droplets in avocado wastewater (AWW) (**Figure 15**). This finding suggests that there may be potential antifungal activity in

AWW. Interestingly, Leikin-Frenkel and Prusky (1998) and (Domergue, Helms, Prusky, & Browse, 2000) pointed out that specialised idioblast cells present in avocado fruit tissue contain lipid soluble compounds, one of which is persin. During CPAO production, avocado flesh undergoes a malaxing stage where parenchyma cells in the flesh is ruptured to release oil droplets. However, during the malaxing process, idioblast cells are not ruptured. A typical idioblast measures approximately 80 µm in diameter with cell walls that are 4 µm in thickness, in contrast to 2.5 µm in parenchyma cell walls (Cummings & Schroeder, 1942). Furthermore, the idioblast cell wall is reported to contain a primary and tertiary cellulosic wall followed by a secondary suberin layer as opposed to a single cellulosic wall found in parenchyma cells (Platt & Thomson, 1992; Platt-Aloia et al., 1983). Research by Yang et al. (2018) also showed that idioblastic cells found in AWW were intact. Hence, any persin in the idioblastic cell would not have been likely to release into the AWW.

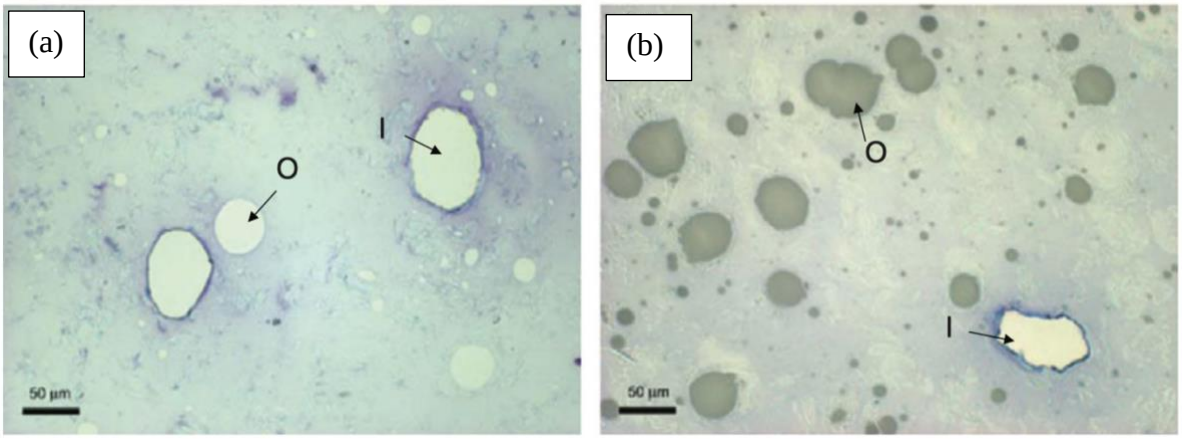


Figure 24. AWW from decanter (c, d) examined using light microscopy. Image (c) was embedded in LR White resin and image (d) was embedded in Spurr's resin. O, oil droplets from parenchyma cells; P, parenchyma cells; I, idioblast cells. Images adapted from (Yang et al., 2018).

7.6.4 Potential antifungal properties in avocado wastewater (AWW)

CPAO utilises matured avocado fruits that are identified as second-grade (non-export quality). There is often a surplus of good quality avocado fruits that are classified as 'second grade' due to its appearance (Eyres et al., 2001). There are limited studies on AWW, the largest by-product of CPAO production. Permal, Leong Chang, et al. (2020) found that AWW could be spray dried into storable powder, possessing high antioxidants and total phenolic content and was found to be effective in preventing lipid peroxidation in cooked pork sausages and pure pork fat. The authors reported that the degree of lipid oxidation was comparable to synthetic additives such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and sodium erythorbate (E316).

Approximately 10% of avocado skin is included in CPAO production to boost nutritional value and visual appearance (Costagli & Betti, 2015; Wong et al., 2011). Therefore, there is a potential for AWW to exhibit antifungal properties in the form of persin from avocado skin. However, as matured fruits are being used in CPAO extraction, it should also be noted that the concentration of persin in the matured avocado skin will be significantly lower. With research data showing AWW to contain anti-fungal idioblast cells, coupled with the inclusion of its protective outer peel, it is likely that the AWW may contain some antifungal properties. Therefore, the aim of this study was to test the antifungal properties of AWW focusing primarily on inhibition of common food borne fungal growth from bread and cheese.

7.6.5 Materials and Method

7.6.5.1 Isolation of mould

To obtain isolate of mould for antifungal assays, Colby cheese and Homebrand white bread was placed inside separate sealable bags, all were purchased from Countdown, New Zealand. The plastic bags were left by the windowsill at ambient temperature until observable mould growth occurred.

After 10 days, a sterilised inoculation rod was used to separately transfer mould from cheese and bread samples onto Sabouraud Dextrose Agar (SDA) plates, purchased from Fort Richards Laboratories, New Zealand. Mould was transferred by dipping the inoculation rod at three different areas on the SDA plates. Once transferred, the plates were then placed into a temperature-controlled oven set to 26˚C for 3 days. After a 3-day incubation period (**Figure 16**), the isolated mould from SDA plates of cheese and bread samples were transferred in a similar manner described above, onto separate SDA plates. These moulds were visually identified in accordance to the physical characteristics outlined by Singhal and Kulkarni (1999). The growth of *Rhizopus nigricans* in bread samples was identified by the presence of white cottony mycelium and black sporangia. Additionally, *Aspergillus niger* was recognised by greenish black conidial heads and yellow pigment diffusing into the SDA and similarly cheese isolates were black mould, or *A. niger*.

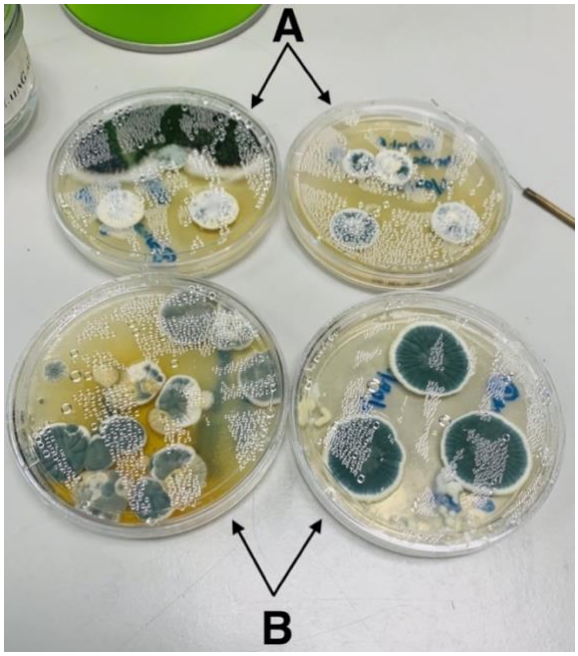


Figure 25. Growth of mould collected from cheese (A) and bread samples (B) after inoculating into SDA and incubating at 26˚C for three days.

7.6.5.2 Antifungal activity of AWW against isolated mould

The bread and cheese mould grown from *section 2.1* was scraped using a sterilised inoculation rod and dipped directly into three different areas of the SDA plates. Four solutions were used to test the antifungal activity of AWW. For the first SDA plate, spray dried AWW powder was diluted at a 2:4 ratio of AWW powder and distilled water. Distilled water was used as a control and bleach (sodium hypochlorite, purchased from New World, NZ) was used as a positive control. For the second SDA plate, the positive and negative

controls were kept the same, except instead of using AWW powder, unprocessed AWW was used instead. Sterile disks were dipped into each solution and then placed onto the SDA plates in between where the mould species were inoculated. The plates were then left to incubate at 26˚C and visually monitored over 3 days to observe growth of bacteria as mentioned in ***Section 2.1***.

7.6.6 Results and discussion

Mould growths, or colonies generally start to grow after 24 to 48 hours. **Figure 17** depicts that there was hardly any growth on day 1. Yet, on days 2 and 3 (**Figures 18** and **19** respectively) there was a burst of visible mould growth of samples from cheese and bread samples. Interestingly, the results from this trial indicated that AWW powder and unprocessed AWW were not successful at inhibiting the growth of mould isolated from bread and cheese samples. The topside view from **Figure 19** indicated that bread samples had visible growth of *Rhizopus nigricans*, and *Aspergillus niger* and additionally mould isolates from cheese only showed growth of *A. niger* (Singhal & Kulkarni, 1999). From **Figures 18** and **19**, bleach (positive control) was successful in inhibiting mould growth. Alternatively, neither unprocessed nor processed AWW supressed the growth of mould isolated from cheese or bread samples. Therefore, no further investigation was carried out.

The novelty of this experimental design is that this is the first time antifungal properties of AWW were tested against *Rhizopus nigricans* and *Aspergillus niger*, both common moulds found in foods (Xing, Li, Xu, Yun, & Lu, 2010). Previously, anti-fungal properties of the avocado fruit have mainly focused on persin its suppression of *C. gloeosporioides*. Anthracnose caused by *C. gloeosporioides* is recognised as the most important disease of the avocado fruit. The mould infects the avocado peel during growing period but the infection remains latent. During fruit ripening and harvest these infections become active and result in

extensive damage to the avocado flesh (Prusky et al., 1982; Sivanathan & Adikaram, 1989) Prusky et al. (1982) successfully isolated persin from the avocado peel and found that a solution containing 790 µg/g persin caused complete inhibition *C. gloeosporioides* germination. Yet, Sivanathan and Adikaram (1989) argued that the resistance of avocado fruit tissue to *C. gloeosporioides* is due to three other antifungal compounds (which were named AvI, AviIII and AvIV) rather than persin (AvII) alone. However, both Sivanathan and Adikaram (1989) and (Prusky et al., 1982) agreed that persin had the most influence on inhibiting growth of the pathogen.

Alternatively, Chia and Dykes (2010) reported extracts from the avocado seed to inhibit growth of a variety of bacteria. Specifically, *Salmonella* Enteritidis, a common bacterial disease of poultry that is the leading cause of gastroenteritis in humans. *Citrobacter freundii,* an organisms found in soil, water and intestinal tract of animals and humans. *C. freundii* has been associated with gastroenteritis, neonatal meningitis, and septicemia. *Pseudomonas aeruginosa*, which is widely distributed both in and out of hospitals and is found in water or contaminated aqueous zones. The pathogen is well known to cause lung infections among patients with cystic fibrosis and serious infections in critically ill patients. *Enterobacter aerogenes* is another bacterium found in hospital wards and has been isolated from the human respoiratory, urginaly, blood and gastrointestinal tract. *E. aerogenes* proves to be a serious issue as aquires numerous genetic mobile elements that strongly contribute to antiobiotic resistance (Adhikari et al., 2022; Davin-Regli & Pagès, 2015; Díaz, Mora, del, & Vidal-Cortés, 2022; Ranjan & Ranjan, 2013). Chia and Dykes (2010) reported ethanol extracts from avocado seeds displayed inhibiting effects on *Salmonella* Enteritidis, *Citrobacter freundii, Pseudomonas aeruginosa and Enterobacter aerogenes* activity at minimum inhibitory concentrations (MIC) of 125, 208, 208 and 250 µg/mL.

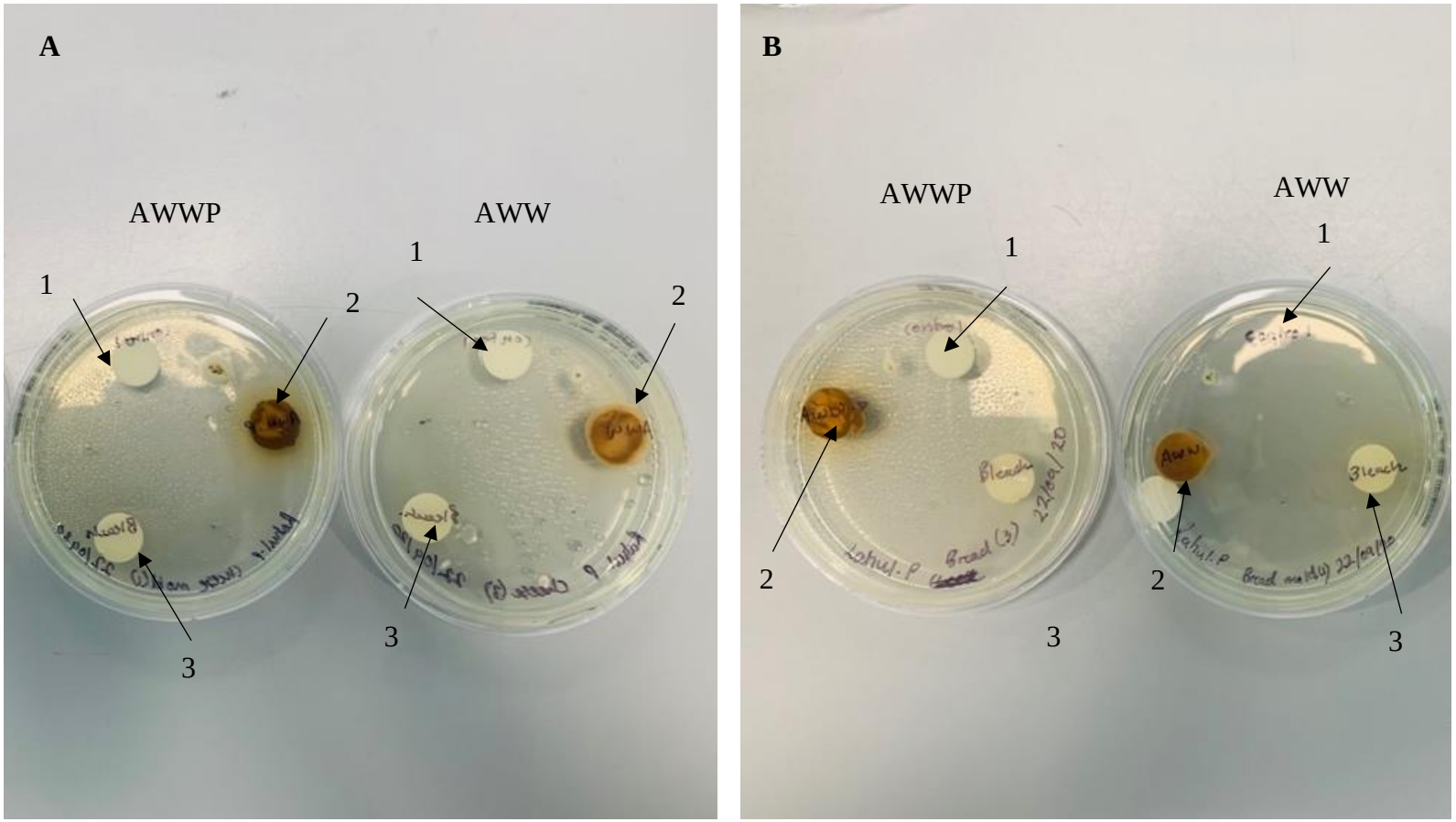


Figure 26. Day 1 of microbial growth from cheese (A) and bread (B) samples inoculated into SDA and stored at 26˚C, *n = 3*. Avocado wastewater powder (AWWP), avocado wastewater (AWW). Disks are labelled control (1), AWW/AWWP (2), and bleach (3).

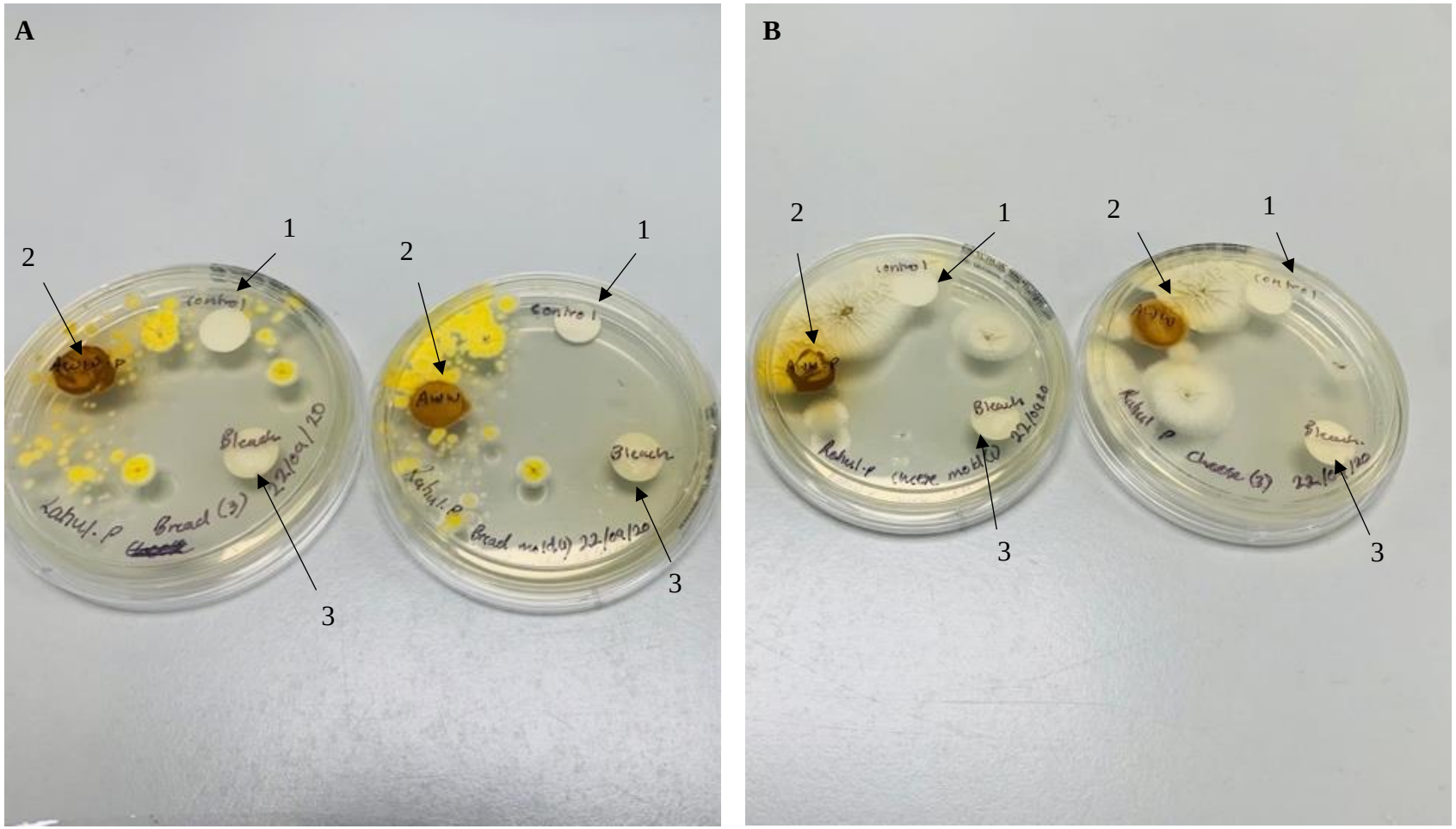


Figure 27. Day 2 of microbial growth from bread (A) and cheese (B) samples inoculated into SDA and stored at 26˚C. Visible microbial growth can be seen on both bread and cheese samples, *n = 3*. Avocado wastewater powder (AWWP), avocado wastewater (AWW). Disks are labelled control (1), AWW/AWWP (2), and bleach (3).

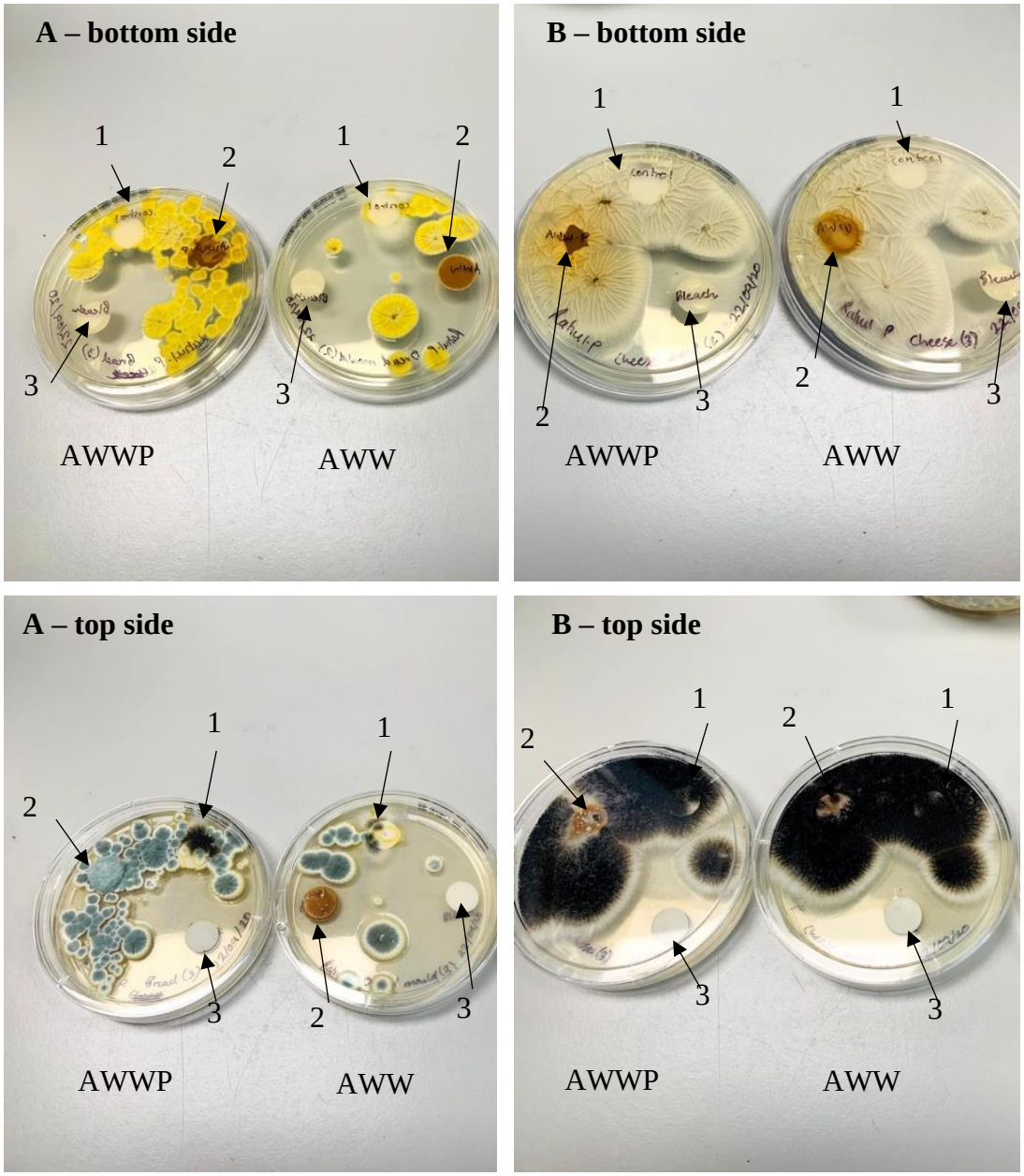


Figure 28. Day 3 of microbial growth from bread (A) and cheese (B) samples inoculated into SDA and stored at 26˚C. Visible microbial growth can be seen from the bottom and top side view of the SDA plates from both bread and cheese samples, *n = 3*. Avocado wastewater powder (AWWP), avocado wastewater (AWW). Disks are labelled control (1), AWW/AWWP (2), and bleach (3).

7.6.7 Conclusion

AWW and spray dried AWW powder was tested for antifungal properties specifically to inhibit growth of mould from cheese and bread samples. Both AWW and spray dried AWW powder was not successful in preventing the growth of *Rhizopus nigricans and Aspergillus niger*. It could be that the antifungal properties in AWW did not target mould from bread and cheese or that there could have been a loss antifungal activity, specifically, persin, due to maturity of the avocado utilised in CPAO. Furthermore, it is known that the idioblast cells found in AWW remain intact, hence, anti-fungal compounds contained inside the oil filled cell did not have the chance to leak into the AWW and interact with growing mould.

7.7 Converting Avocado Skin into Biodegradable Plastic Film

7.7.1 Introduction

The most abundant and renewable biodegradable material available from nature is cellulose with around 700 billion tonnes produced annually (Q. Xu, Chen, Rosswurm, Yao, & Janaswamy, 2016). Cellulose has good biodegradability. A study on the biodeterioration of man-made textiles found that 12 samples (1cm x 1cm) of cellulose acetate fibres significantly deteriorated after 2 months in moist soil and was completely destroyed after 4-9 months (Northrop & Rowe, 1987; Puls, Wilson, & Hölter, 2011).

At present, the development of safe and sustainable packaging is of major concern for the food industry with the main aim to preserve food and minimise waste throughout the distribution chain. Most food packaging is made from petroleum-based plastics, accounting for a production of approximately 350 million tonnes per year (Haghighi, Licciardello, Fava, Siesler, & Pulvirenti, 2020). An important factor to consider is that, once packaging materials have reached the end of their useful life they should ideally, degrade within a reasonable amount of time without negatively impacting the environment (Garrido-Romero, Aguado, Moral, Brindley, & Ballesteros, 2022). Yet, petroleum based plastics can take an extremely long period to decompose, with some research estimating periods of up to 500 years (Rajmohan, Ramya, Viswanathan, & Varjani, 2019). A substitute to petroleum-based plastics is utilising cellulose-based packaging due to its low toxicity and biodegradable nature (Edgar et al., 2001; Q. Xu et al., 2016).

Cellulose can be extracted from various sources like cotton, wood, hemp, tunicates or microorganisms, and most plant-based materials (Garrido-Romero et al., 2022). It is already utilised to make various items such as food containers, carboard boxes and most commonly,

paper. The challenge in utilising cellulose is its insoluble nature in water, common organic solvent, and inorganic liquid. Cellulose (**Figure 24**) is a polysaccharide made up of β-1-4-linked D-glucopyranosyl units, that form a ridged network structure through strong intra- and inter- chain hydrogen bonds (Figueiredo, Ismael, Anjo, & Duarte, 2010). A structural arrangement such as this results in water insolubility characteristics, limiting a number of potential applications for cellulose. Q. Xu et al. (2016) explained that several solvent systems have been explored to overcome cellulose insolubility. Some of which include, cadoxen, cuen, ammonium, calcium and sodium thiocyanate, and ionic liquids such as lithium chloride/1,3-dimethyl-2-imidazolidinone (LiCl/DMI). However, the issues of toxicity, environmental pollution, limited resource, and high-power consumption involved in utilising these solvent systems make them unattractive for large scale operations.

Currently, industrial scale operations have focused on developing processes that are non-polluting and targeted towards environmental conservation. Therefore, 'green chemistry' techniques, such as ionic liquids (1-butyl-3-methylimidazolium chloride ([BMIM]CL)) have also been researched in dissolving cellulose. Ionic liquids are designated 'green' solvents as they are non-flammable, thermally and chemically stable and have extremely low vapor pressure. However, high production costs and moisture sensitivity impair their complete utilisation (El Seoud, Koschella, Fidale, Dorn, & Heinze, 2007; A. Xu, Wang, & Wang, 2010; Q. Xu et al., 2016). Efforts to solubilise cellulose by Q. Xu et al. (2016) involved incorporation of a new solvent system, utilising common salts. The authors found that utilising zinc chloride solution was an effective solubilizer with enhanced gelling properties in the presence of calcium chloride. The zinc and calcium ions create nano fibrils by crosslinking cellulose chains producing strong biodegradable films. Utilising inorganic salt solutions to solubilise cellulose is favourable as it is cost-effective, recyclable and most importantly a 'green' process.

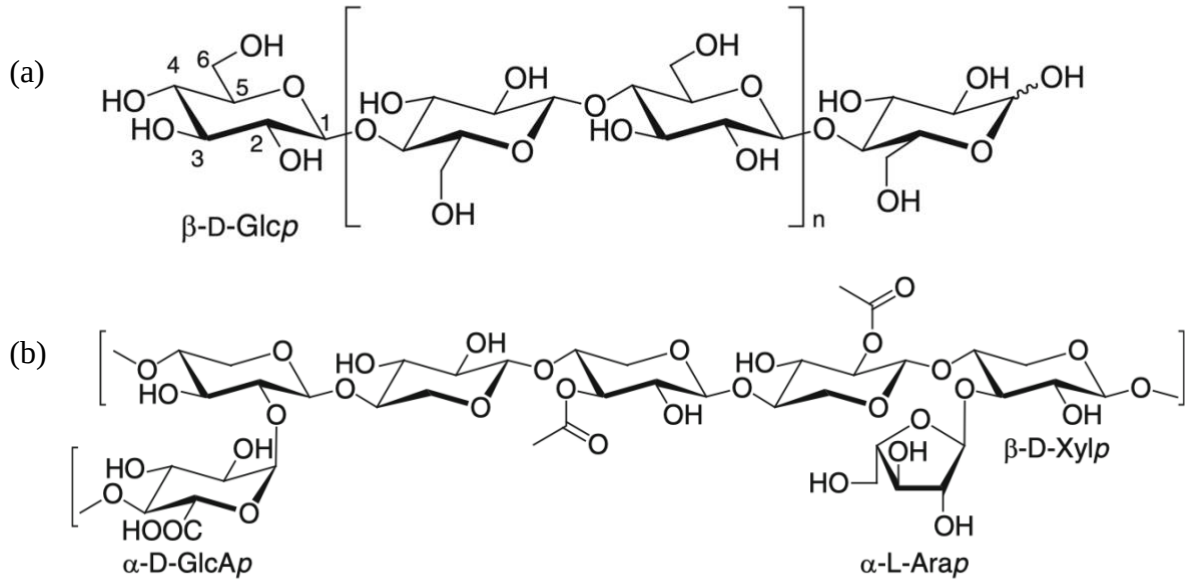


Figure 29. Structure of cellulose (a) and hemicellulose (b).

An unrealised source of cellulose, currently going to waste, is in the production of cold pressed avocado oil (CPAO). Research by Permal, Leong Chang, et al. (2020) showed that for every 70 kg of avocado oil that is produced, approximately 153 ± 5 kg of avocado skin waste is generated. The authors reported that approximately 20.1 ± 0.5% (wet basis) of the skin was composed of mainly non-digestible carbohydrates. Dávila, Rosenberg, Castro, and Cardona (2017) found that of the total non-digestible carbohydrates in avocado peel, around 27.58 ± 1.18% and 25.30 ± 1.24% (wet basis) was cellulose and hemicellulose respectively. As avocado skin is abundant in non-digestible carbohydrates, mainly cellulose (Dávila et al., 2017; Permal, Leong Chang, et al., 2020), the aim of this research was to determine if cellulose in avocado skin could be solubilised and converted into biodegradable plastic using inorganic salts as proposed byQ. Xu et al. (2016). Although avocado seeds has already been

successfully converted into biodegradable plastic (Maulida Lubis, Harahap, Ginting, Sartika, & Azmi, 2016), avocado skin has yet to be exploited for its renewable cellulose content, which is where the novelty in this research lies.

7.7.2 Materials and Method

7.7.2.1 Raw Materials

Avocado skin from the *Hass* variety was collected from the CPAO processing plant at Olivado Ltd in triplicates on three separate production days within a week. The skin was collected from the destoner and placed into a 26 cm x 38 cm snap lock plastic bag (Glad, Australia). The samples were stored at 4˚C in an ice bath and transported to the laboratory at Auckland University of Technology, New Zealand within 3.5 h. The skin was then freeze-dried using the Alpha 1-2 Dplus Laboratory Freeze Dryer for 48h at -75˚C and 1 x 10^{-3} mbar, and then stored at -18˚C until required for plastic production.

7.7.2.2 Chemicals

Microcrystalline cellulose, zinc chloride ($ZnCl_2$) and calcium chloride ($CaCl_2$) were purchased from Sigma-Aldrich, New Zealand. Ethanol and glycerol were purchased from Thermofisher, New Zealand.

7.7.3 Preparation of Zn-cellulose solutions with/without $CaCl_2$

Films were prepared as outlined by Q. Xu et al. (2016). Initially the cellulose paste was prepared by adding 1.6 g of distilled water to 0.8 g (3% w/w) of microcrystalline cellulose Then in a separate beaker, $ZnCl_2$ (12.75 g) was dissolved in 6.0 mL of distilled water making a 68% w/w solution. The solution was then equilibrated at 65˚C (±1) for 10 minutes in a water bath. Afterwards the $ZnCl_2$ solution was carefully poured into the cellulose paste and

mixed thoroughly for 30 minutes. For the Ca-Zn solution, 0.031 g of $CaCl_2$ was mixed with 12.75 g of 68% ZnCl2 (w/w) solution to produce a $CaCl_2$ molar ratio of 0.03 to $ZnCl_2$. The solution was mixed at 65˚C for 10 minutes. Then 3% cellulose paste was mixed in with the $ZnCl_2$ and $CaCl_2$ solution at 65˚C for 30 minutes.

Both Zn-cellulose and Ca-Zn-cellulose solutions were casted on glass petri dishes and immersed in 500 mL of absolute ethanol. The films were left to coagulate for 30 minutes after which the ethanol was drained and again submerged in a fresh ethanol bath for another 30 minutes. The film was then left on a plastic frame and air dried at room temperature overnight. After drying, the film was placed in a water bath for 30 minutes to remove any excess salt and subsequently soaked in glycerol (5% v/v) for another 30 minutes. Finally, the film was air dried producing the finished product.

7.7.4 Preparation of Zn-avocado skin solution with the addition of $CaCl_2$

Firstly, the freeze-dried avocado skin was removed from the freezer and blended into fine powder using the magic bullet blender (MBR-2101, USA) The procedure to produce the avocado film was described in Section 2. In place of cellulose, avocado skin powder was used instead. Three sets of avocado powder samples were made. The first sample (a) included direct substitution with 0.3 g dried peel (3% of paste solution - like Section 2), the second sample (b) included 3.7 g of dried peel assuming the non-digestible carbohydrate is equivalent to cellulose, and the third sample (c) included 7.4 g of dried peel by considering the actual cellulose content. The second and third samples were prepared based on the calculation of cellulose content in avocado peel as reported by Dávila et al. (2017).

7.7.5 Results and discussion

7.7.5.1 Cellulose-based plastic

Following the method outlined by Q. Xu et al. (2016), a cellulose-based film was successfully replicated in the lab. The Zn-cellulose films were white in colour and with the addition of Ca^{2+} ions, a transparent film was produced. In theory, salt ions (Zn^{2+} and Ca^{2+}) promoted the breakdown of tight cellulose network structures, increasing solubility, and encouraging crosslinking of cellulose nano fibrils. Research on three-dimensional structural analysis has revealed that cellulose adapts a rigid 2-fold helical structure with strong intra-chain O3H · · ·O5 hydrogen bonds (Figure 24) (Langan, Nishiyama, & Chanzy, 1999; Nishiyama, Langan, & Chanzy, 2002).

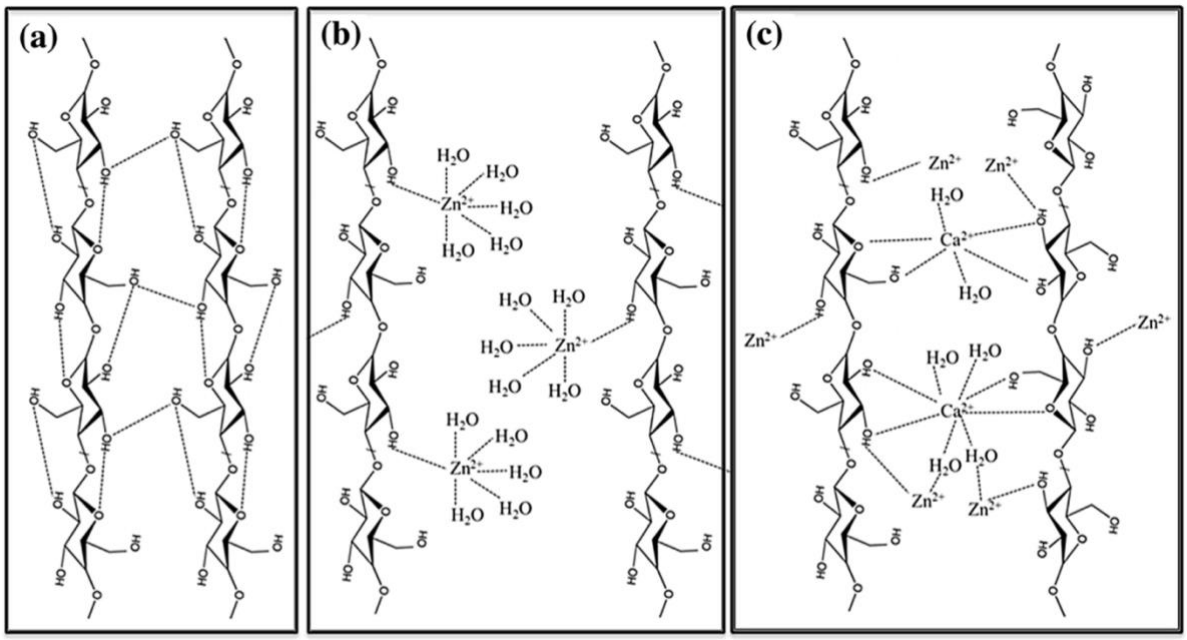


Figure 30. Depiction of the interaction between a pair of cellulose chains. (a) Native cellulose chains held together through strong hydrogen bonding (broken lines). (b) Zn^{2+} ions bound to O3H break the canonical O3H · · ·O5 hydrogen bonds. As a result, the cellulose chains gain flexibility, allowing water molecules (H_2O) to penetrate the network and lead to cellulose dissolution. (c) Subsequent addition of Ca^{2+} ions crosslink free Zn-cellulose chains through cooperative interactions between the hydroxyl groups, Zn^{2+} ions and water molecules, resulting in the formation of nano fibrils and gelation. This regenerated cellulose structure is responsible for the high tensile strength of films (Q. Xu et al., 2016).

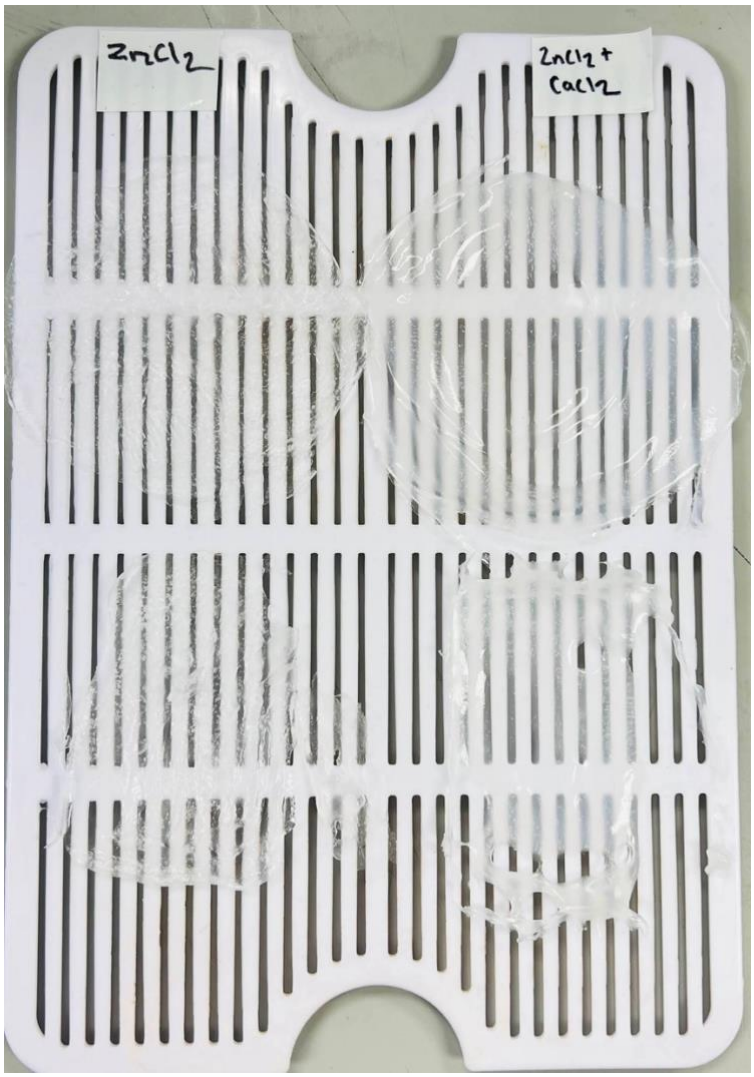


Figure 31. Comparison of cellulose and salt films using 3% cellulose mixed with 68% salt solution of $ZnCl_2$ (left) and $ZnCl_2$ + $CaCl_2$ solution (right).

Q. Xu et al. (2016) explained that bacterial polysaccharides such as welan, xanthan and acetan results in the substitution or breakdown of O3H · · ·O5 hydrogen bonds (**Figure 25**), which lead to a 3-fold helical structure (Rengaswami Chandrasekaran, Janaswamy, & Morris, 2003; R. Chandrasekaran & Radha, 1997). The abandonment of one or more of the O3H · · ·O5 bonds in the cellulose stems adds flexibility, producing water soluble cellulosic polysaccharides. Q. Xu et al. (2016) suggests that a similar phenomenon occur in the Zn-cellulose complexes, specifically Zn^{2+} ions pairing with the O3H atoms in forming the Zn · · · O3H interactions and disrupting the inter-chain hydrogen bonds leading to flexibility. The

resulting non-rigid molecular network are susceptible to water penetration and hence increases the solubility of cellulose. Although uncertain, the authors hypothesised that with the addition of Ca^{2+} ions, non-rigid Zn-cellulose chains may be crosslinked effectively through Ca^{2+} ions forming nano fibrils to create a stronger and visibly more transparent film. However, with the addition of Ca^{2+} ions, the film was less transparent than the sample using Zn^{2+} ions only (**Figure 25).** Additionally, the film was not ductile or durable and was easily cracked upon applying pressure.

7.7.5.2 Producing avocado peel-based plastic

Figure 26 shows that utilising $ZnCl_2$ and $CaCl_2$ solution was the most effective in producing a transparent film. Therefore, when utilising avocado skin (**Figure 27**), only a Ca-Zn-avocado skin mixture was utilised in creating the film. As expected, the film retained a brown colour. Additionally, the Ca-Zn-avocado skin film was not as strong compared to the films created using pure cellulose in *Section 3.1*. As seen in **Figure 28** gentle handling of the film resulted in a tear around the bottom left-hand side. It can also be seen in **Figure 28** that with the addition of higher amounts of avocado peel (specifically *samples b and c*) the film did not remain intact.

Figure 32. Conversion steps of avocado peel into film. Freeze dried avocado skin (A), Powdered freeze-dried avocado skin, mixed with 1.6 g distilled water (B), and film created using Ca-Zn- avocado skin mixture (C).

Figure 33. Avocado film created using 0.3 g dried avocado peel (a), 3.7 g dried avocado peel (b) and 7.4 g dried avocado peel (c).

Supplementary Information

With regards to colour and uniformity, the film quality was not as satisfactory compared to the cellulose-based film. Prior to processing avocado powder there was no bleaching or filtering process. Hence, the brown colours and particulate matter can be seen on the film. Previous methods of decolouration have incorporated the use of 4% soapnut solution to avoid chlorinated bleaching agents for natural fibres. Since soapnut does not contain artificial whiteners or softeners, it is deemed more environmentally friendly as the saponin acts as an effective bleaching agent (Kose & Bayraktar, 2016; Reshmy, Philip, Vaisakh, et al., 2021). Furthermore, biodegradable fibres can then be bleached using 80% acetic acid, 45% nitric acid and concentrated sulphuric acid (Reshmy, Philip, Paul, et al., 2021; Sun & Tomkinson, 2005). Although harsher, these methods are more effective at removing the remaining residue and colour residues in the organic fibres leaving behind only the cellulose. Essentially these methods were not explored in this study because of efforts to minimise environmental impact and to reduce processing cost and time.

Research has shown that the avocado peel contains similar amounts of cellulose and hemicellulose, approximately 27.58 ± 1.18% and 25.30 ± 1.24% (wet basis) respectively (Dávila et al., 2017). While cellulose is crystalline, strong, and resistant to hydrolysis, hemicellulose is composed of random amorphous structures and has a low molecular weight. As a result, hemicellulose has little strength compared to cellulose (Hosseinaei, Wang, Enayati, & Rials, 2012). With regards to use as packaging material, hemicellulose exhibits poor compatibility with thermal stability than traditional plastics. Moreover, a large number of hydrophilic hydroxyl groups on the side chains of hemicellulose render the film susceptible to absorption of moisture, producing poor performance when used in a humid environment (Nešić et al., 2019). Alternatively Zhao, Sun, Yang, and Weng (2020) argued that hemicellulose (**Figure 1**) has a unique advantage in that its functional hydroxyl group and various structures can be easily modified to meet different requirements. Hence

adjustments can be made to improve the performance of hemicellulose films intended for packaging material through physical and chemical modifications. The improvements include changing the microstructure and composition of hemicellulose films through blending, addition of plasticizers i.e. glycerin, xylitol, and sorbitol, and addition of reinforcing agents such as quaternized hemicellulose (Guan et al., 2014; Mikkonen, Heikkilä, Willför, & Tenkanen, 2012). Alternatively research by Reshmy, Philip, Vaisakh, et al. (2021) suggests to either wash out or convert hemicelluloses into pure nanocellulose using acid wash and bleaching. Therefore, the Ca-Zn-avocado skin film may not have been as strong as the cellulose film due to the high ratio of hemicellulose and cellulose in the avocado skin itself. This is evident in **Figure 24** where a higher concentration of avocado peel in *sample b* and *c* resulted in poor formation of film compared to *sample a.* Further processing to remove hemicellulose would therefore be a viable option to improve the performance of the Ca-Zn-avocado skin film.

7.7.6 Conclusion

A biodegradable film was prepared using Zn^{2+} ions. The Zn^{2+} ions solubilised cellulose found in avocado skin by breaking O3H · · ·O5 hydrogen bonds responsible for keeping an insoluble and tight cellulose network structure. The addition of Ca^{2+} ions added crosslinks to the Zn-cellulose chains to form nano fibrils that produces a film. However, the film was not as strong as anticipated presumably due to the presence of hemicellulose in the avocado skin that was not removed. Utilizing inorganic salt solutions to solubilize and crosslink cellulose stands out as a viable option to valorise avocado peel waste from CPAO processing into biodegradable film. Future developments utilising cellulose-based films from organic waste to produce a biodegradable film would be beneficial due to its recyclable nature and most importantly being a “green” process method.

8 References

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