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Abstract

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).

Keywords

Avocado seed valorisation; Friction cooker; Toxins in avocado seed; Persin extraction; Waste valorisation

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, Seale, Hamid, and Kam (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, Mazzola, Shegog, Ziegler, and Lambert (2019) isolated perseorangin, a natural colourant from the avocado seed for potential use as dye. Perea-Moreno, Aguilera-Ureña, and Manzano-Agugliaro (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 1),** 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).

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 in 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.

2 Materials and Methods

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 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.

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). Apricot seeds were packed by Waitaki Orchards

126 Ltd, and purchased from Bidfood, a food wholesaler in Dunedin, New Zealand. Brown rice
127 was purchased from Davis Trading Co, New Zealand. Barley (Gladfield Pilsner malt) was
128 purchased from Home Brew West, New Zealand.

129 Apricot seeds, as well as avocado seeds and leaves were freeze-dried using the Alpha 1‑2
130 LDplus Laboratory Freeze Dryer for 48 h at −65 °C and 1×10^{-3} mbar. Another group of
131 avocado seeds were oven-dried (70 °C) using the Piron PF4005D oven (Italy), until the seeds
132 reached a consistent weight (approx. 3 days). Apricot seeds, avocado leaves, and a portion of
133 both freeze-dried and oven-dried avocado seeds were finely ground and stored in the freezer
134 (–20 °C) before analysis. The remaining portion was crushed using a grain mill grinder with
135 roller gap set at 5 mm for friction cooking or stored at –20 °C until required.

136

137 ### *2.3 Making extruded RTE snacks using the friction cooker*

138 Brown rice, barley, freeze-dried and oven-dried avocado seed were extruded using a single
139 screw, Zapmill™ Friction Cooker (Auckland, NZ) through a 5mm die opening (refer to
140 Supplementary Information S1.2 for a visual representation). The feed flow rate was fixed at
141 5.3, 13.1 and 12.8 kg/h for avocado seeds, brown rice, and barley respectively. The screw
142 shaft rotation speed was maintained at 50 Hz. The extruded snacks were air-dried at room
143 temperature for 30 minutes to prevent them from sticking together and stored in airtight
144 containers. The heat generated from friction cooking can elevate the temperature to 130 °C at
145 the exit of the barrel.

146

147 ### *2.4 Physical analysis of extrudates*

148 #### *2.4.1 Lateral expansion ratio*

149 Lateral expansion (LE) ratios were calculated as outlined by Ainsworth, İbanoğlu, Plunkett,
150 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 (1) below:

$$LE = \frac{diameter\ of\ extrudate - diameter\ of\ die\ hole}{diameter\ of\ die\ hole} \times 100 \quad (1)$$

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:

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

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.

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).

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).

$$\text{Porosity} = \frac{bulk\ volume - apparent\ volume}{bulk\ volume} \quad (3)$$

Where: Bulk volume = $1/\rho_b$ (4)

and Apparent volume = $1/\rho_s$ (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 50 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.

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 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, 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. 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.

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

The Folin-Ciocalteu's (F-C) assay was conducted as outlined by Singleton, Orthofer, and Lamuela-Raventós (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).

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 (99.12% purity) standard 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 → 323 (quantifier ion), 221 (qualifier ion) with a fragmentor voltage of 220 V and collision energy of 3 eV and 8 eV, respectively. The relative abundance was 17% and was within the 20% acceptance criteria. The matrix effect was calculated at 98.2%, showing little ionization suppression. Therefore, matrix matching calibration was not necessary. The chromatographic repeatability (n = 6) was determined and the residual standard deviation (RSD), limits of detection (LOD) and limit of quantification (LOQ) were calculated as outlined by Permal, Chang, et al. (2020). RSD, LOD and LOQ were 10.4%, 0.04 µg/mL and 0.15 µg/mL respectively.

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 ^{1}H NMR. The crude sample was further purified by flash column chromatography (silica gel) using

10% EtOAc in CH_2Cl_2 as the eluent, affording persin as a colourless oil. This purified sample was analysed by ^{1}H and ^{13}C NMR using a Bruker Ascend spectrometer operating at 400 MHz for ^{1}H nuclei and 101 MHz for ^{13}C nuclei. The NMR spectra can be found in Supplementary Information SI.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 performed according to the set-up described in *section 2.7*.

A Poroshell 120 EC-C18 (2.1 x 50 mm, 2.7 µm) column was used for the analysis of persin. The mobile phases were composed of water containing 0.1% (v/v) formic acid (A) and acetonitrile containing 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. The drying gas (N_2) temperature was set to 300˚C with a flow rate of 10 L/min; the vaporiser temperature was set to 250˚C and a nebuliser pressure of 30 psi was used. A positive ion mode was used with MRM for quantitative analysis. The precursor-to-product ion transition used for persin was [M+H] + m/z 381 → 285 (quantifier ion), 363 (qualifier ion) with a fragmentor voltage of 100 V and collision energy of 1 eV and 4 eV, respectively. The relative abundance was 55% and was within the 20% acceptance criteria.

Chromatographic repeatability (n = 10) was estimated and the RSD, LOD and LOQ was calculated as 6.8%, 0.07 µg/mL and 0.25 µg/mL respectively.

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.

3 Results and discussion

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 1**) 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, Barbosa-Martín, Martínez-Antonio, González-Mondragón, & Betancur-Ancona, 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 4**). 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. 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; 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 1**). 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).

348 Therefore, the high porosity in avocado seed extrudate was attributed to longitudinal
349 expansion along the axis of extrudate flow as opposed to lateral expansion.
350
351 *3.2 Textural analysis of extrudates*
352 Avocado seeds were significantly lower in hardness (4.34 N/cm^2, $p < 0.05$) compared to
353 brown rice and barley (12.2 N/cm^2 and 10.7 N/cm^2 respectively). The avocado seed extrudate
354 was also lower in crispiness (1.4 N/mm, $p < 0.05$) compared to brown rice and malt barley
355 8.7 N/mm and 7.9 N/mm respectively). For both hardness and crispiness, brown rice and malt
356 barley were not statistically different from each other ($p > 0.05$).
357 Kumar, Xavier, Lekshmi, Balange, and Gudipati (2018) suggested that the hardness of
358 extrudates correlates with fiber content. The ability of fiber to bind to water more strongly
359 than starch inhibits water loss at the die and hence reduces the ability for extrudate
360 expansion. The current study found a significant decrease in hardness for avocado seed
361 extrudate, which is composed of approximately 19.5% (w/w) fiber (Permal, Leong Chang, et
362 al., 2020). Brown rice and barley on the other hand contain around 2.86 % and 20.2% (w/w)
363 total dietary fiber respectively (Djurle et al., 2016; Wu et al., 2013). Therefore, fiber did not
364 dictate the hardness of extrudates in this study. Instead, the level of hardness may be an effect
365 of lateral expansion (%) – avocado seeds had the lowest level of lateral expansion compared
366 to the other two grains.
367 Determination of mechanical strength and stiffness of extrudates involved the measurement
368 of intensive properties such as Flexural Modulus (FM) and Modulus of Rupture (MoR). MoR
369 represents the maximum bending stress of a material before breakage and FM represents the
370 resistance to bending in a material (Callister & Rethwisch, 2014). **Table 1** showed that MoR
371 followed similar patterns to hardness, with avocado seed extrudate having significantly ($p <$
372 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.

3.3 Antioxidant capacity and total phenolics

Table 2 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 2** 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 Wang, Bostic, and Gu (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 2**, 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. Therefore, future work should focus on the organoleptic properties of the extruded avocado seeds.

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.

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 3** (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.

Persin concentration in oven-dried and freeze-dried avocado seeds (**Figure 2)** 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 (refer to Supplementary Information S1.3). Results showed that isolated persin was stable at temperatures up to 130˚C. 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.

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.

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