Chemical Characterization of Avocado Oil to Inform Standard Development and Improve Adulteration Detection

By

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Abstract

Avocado oil is a high-value edible oil rich in monounsaturated fatty acids and phytosterols. As consumers seek out healthier and more heat-stable cooking oil, avocado oil has been growing rapidly in popularity. However, the combination of high demand and lack of standards of identity making it difficult to ensure oils are of the quality and purity advertised on the label. Economically motivated adulteration that goes undetected can cause authentic, good quality products to be outcompeted in the market and risks consumer health if an unlabeled allergen is added. The goal of this work was to better understand the chemical composition of avocado oil, or its "fingerprint" so appropriate standards of identity could be created, and so improved adulteration detection methods could be developed.

The quality and purity of avocado oils available on the United States market were analyzed to identify the biggest issues in the industry. We found that 82 % of avocado oils were either of poor quality (contained high oxidation and/or hydrolyzation products) or were adulterated. Once the need to develop standards was demonstrated, two different grades of avocados from two regions were collected throughout the harvest season and pressed using a laboratory-scale mill to understand how these variables impacted the quality of the oils. Damaged fruits caused a significant increase in free fatty acidity; however, it was also shown that if good quality fruits were used extra virgin oil could be made from either the whole fruit or the mesocarp. The oils described above plus an additional experimental design that incorporated avocado cultivar assessed how purity parameters vary within authentic avocado oils. Region and harvest time contributed to the largest significant differences in fatty acids and sterols profile. Standards need to be wide enough to accommodate different producing regions, which now includes the United States, Kenya, Peru, and South Africa in addition to New Zealand and

Mexico while still minimizing the chance for adulterations to go undetected. New methods using triacylglycerols and cis-vaccenic acid were developed to help support adulteration detection in avocado oil. Cis-vaccenic acid has a higher concentration in avocado oil compared to high-oleic seed oils, which are traditionally difficult to detect in avocado oil, and can be used as a marker of adulteration. By combining triacylglycerol analysis with principal component analysis, adulteration can be detected based on the location of an unknown on the plot, providing an alternative way to detect purity without traditional standards. The findings in this work contribute to ensuring the purity, quality, and safety of avocado oils; help regulators and policy makers to better protect consumers; and support the growth of authentic avocado oil sectors.

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Chapter 1

Introduction

Avocado oil is a new high-value edible oil on the market. In 2021 the United States imported the highest number of avocados on record; 2.7 billion pounds, a significant increase from the beginning of the 2000s, where imports were under 1 billion pounds (Kramer, 2022). The increased importation led to the expansion of consumption of avocado related products; avocados that are not fit to be sold as fresh fruit are made into processed products, like avocado oil (Wong, 2010). The rise in avocado oil popularity is also related to the health benefits associated with it, which like olive oil, is high in monounsaturated fatty acids and antioxidants (Fernandes et al. 2018, Wang et al. 2019, Wong et al. 2010). Thus, avocado oil has some of the same nutritional value as consuming the fresh fruit but can help reduce food waste by using fruits that could not be sold in stores. There is significant potential for avocado oil, but the high demand, unstable supply chain and rapid growth of the industry puts this new product at increased risk for adulteration.

High-value food products are common targets of food fraud with olive oil leading with the highest instances of fraud, followed by milk, honey, and saffron (Moore, et al. 2012). Edible oils are at particular risk due to the ease of mixing other oils not on the label without the consumer noticing. This practice is called economically motivated adulteration and is often done to raise the profit margin (U.S. Food and Drug Administration, 2021). These adulterated oils can then be sold for less money, which causes extreme damage to the market as they can outcompete the pure, authentic oils. Consumer health is also at risk; a 1990s operation used Turkish hazelnut oil to adulterate bottles labeled 100 % olive oil, a significant tree nut allergy concern (Muller, 2007). Although it can be difficult to keep up with food fraud in the oil industry, two of the most

effective ways are to develop standards of identity, a set of chemical parameters that are unique to each oil, to regulate the quality and purity of the product and develop new methods to better detect the presence of adulterants in oil. At the time of the 1990 hazelnut oil incident, olive oil already had established standards of identity through the International Olive Council (IOC), which made it easier to determine how hazelnut oil was chemically different from olive oil and methods were developed to help better differentiate these two products (Azadmard-Damirchi, 2009; Lopez-Diaz, 2003; Ruiz del Castillo, 1998). Avocado oil, however, currently has no established standards; the lack of regulation combined with the lack of knowledge on the chemical composition of authentic avocado oil makes adulteration detection even more difficult.

The central hypothesis of this work was that understanding the chemical composition of authentic avocado oil will aid in standard development and can be utilized to develop methods to detect adulteration. This hypothesis was tested in three primary aims: 1) identifying the differences in avocado oils currently on the market and to what extent adulteration was occurring; 2) extracting avocado oil using fruits accounting for several parameters (ex. region and harvest time) to determine how they impact avocado oil quality and purity and how the variation seen compares to proposed standards; 3) using the authentic avocado oil samples to develop new methods to detect adulteration.

In Chapter 2, a chemical analysis of avocado oils currently available on the United States market was performed. The quality and purity of the oils was compared to what was advertised on the bottle to determine if any disparities could be found, with the goal of identifying common adulterants so future methods could be developed. This work also aimed to elucidate needed areas of research for avocado oil, including the urgent need for standards of identity.

Due to the lack of authentic reference samples for avocado oil, in Chapter 3, a laboratory-scale mill was used to process avocados into oils. This work focused on determining what variables most significantly impact the quality of the oil, analyzed as the oxidation and hydrolyzation of fatty acids. Fruits were harvested from early to late growing season from two locations: California and Mexico, using two different grades of fruit. It also worked to answer a pertinent question for industry and standard development agencies: can virgin/extra virgin quality oil be made from processing the whole fruit as well as from only the mesocarp.

Chapter 4 used two different experimental designs and extraction techniques to assess the variation in avocado purity parameters according to harvest time, region grown, cultivar, fruit grade, and processing using whole fruit vs mesocarp in the most comprehensive analysis of avocado oil purity parameters to date. The methods used in this study were fatty acid profile and sterols analysis, which are the most common analyses used in standards of identity to confirm oil purity. Each fatty acid and sterol molecule has a typical range of values seen in avocado oil that can be used to create a "fingerprint" to differentiate it from other oils. The goal of this study was to compare the fatty acids and sterols results, as well as tocopherol content, a minor component in avocado oil, to current proposed CODEX standards and propose adjustments.

Cis vaccenic acid (C18:1 n-7) is an isomer of the more common oleic fatty acid (C18:1 n-9). When the fatty acid profile of oils is calculated oleic acid is reported as the sum of these two isomers. Chapter 5 quantifies the amount of cis-vaccenic acid in avocado oil and compares its concentration to other edible oils. It was proposed that this compound has the potential to be a powerful marker in differentiating avocado oil from other high oleic seed oils, which can be difficult to detect. Applying this marker for use in industry is simple, as it can be quantified

using the existing IOC fatty acid profile method. Method validation was done using avocado oil samples blended with varying amounts of high oleic seed oils.

In Chapter 6, a new method using triacylglycerol (TAG) content in combination with principal component analysis (PCA) was developed to detect adulteration in olive oil. TAGs from a representative set of olive oils were analyzed using a UHPLC-CAD (charged aerosol detector) and then plotted using PCA, along with several other likely adulterant oils. Adulteration is determined by the location of a sample on the PCA plot. Method optimization and validation were performed by blindly analyzing blended samples. This study was first developed for olive oil instead of avocado oil because there are still instances of adulteration in the olive oil industry primarily with imported/exported oils and those sold in bulk often used in restaurants (Camin et al. 2016; Bayramer et al., 2018; Esteki et al., 2019; Wang et al. 2012). In addition, there was not only a lack of proposed standards for avocado oil, but a lack of access to authentic avocado oil reference samples needed to validate this method at the time of its development. Although Chapter 6 was the first study to be published, it was included here to best fit within the greater avocado oil story.

Chapter 7 is the application of the tandem TAG and PCA method to avocado oil, which was done once the chemical composition of avocado oil was better understood and a database of pure samples was available. The avocado oils extracted in Chapters 3 and 4 were analyzed using the UHPLC-CAD for their triacylglycerol content and plotted on the PCA along with several other common avocado oil adulterants. The method was validated by testing samples of known composition from fatty acids and sterols analysis. The application of this method to avocado oil is especially useful because not only does it take much less time than fatty acids and sterols

analysis but established limits (maximum and/or minimum) are not needed since adulteration is determined by location of a sample on the plot.

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Chapter 2

First Report on Quality and Purity Evaluations of Avocado Oil Sold in the US

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Abstract

The demand for avocado oil has increased significantly as consumers resonate with its potential health benefits, however, due to the lack of enforceable standards, consumers are unprotected from fraud (i.e., economic motivated adulteration). This study analyzed avocado oils currently on the market in the US to evaluate their quality (e.g., free fatty acidity, peroxide value, UV absorbances, vitamin E) and purity (e.g., fatty acids, sterols, triacylglycerols). Our results showed that the majority of commercial samples were oxidized before reaching the expiration date listed on the bottle. In addition, adulteration with soybean oil at levels near 100% was confirmed in two "extra virgin" and one "refined" sample. These findings demonstrate there is an urgent need to develop standards for avocado oil not only to ensure the consumers receive

high quality and authentic products but to establish a level playing field to support the continuing growth of global avocado oil industry.

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Background

The world's production of avocados increased one million tonnes from 2014 to 2017 and is projected to continue rising with Mexico accounting for one third of the world's production (Altendorf, 2019). Consumer demand for the fruit is largely due to the health benefits associated with avocados, which have high amounts of monounsaturated fatty acids and antioxidants (Fernandes, et al. 2018; Wang et al., 2019; Wong, Requejo-Jackman, & Woolf, 2010). The rising popularity of avocados has also led to the rise in avocado products, namely avocado oil.

Competition in the market place for avocado oil continues with one major boundary, there are currently no standards to determine if an avocado oil is of the quality advertised and authentic. Oils that are of poor quality or blended with cheaper edible oil can be traded and sold at lower prices than high quality or authentic products leaving bulk buyers, food service professionals and consumers unprotected. With no standards available, there is no way to ensure avocado oil is safe. Standards developed for edible oils commonly fall into two categories, quality and purity. Quality can be controlled by the fruit used to make the oil, extraction process, storage; it's mostly related to level of hydrolysis of the fruit and oxidation of the oil (Woolf et al., 2009). An oil is considered pure or authentic if there are no other additives or oils present other than what is listed on the label.

So far, much of literature has focused on improving extraction methods for avocado oil (Corzzini, Barros, Grimaldi, & Cabral, 2017; Dos Santos, Alicieo, Pereira, Ramis-Ramos, & Mendonça, 2014; Krumreich, Borges, Mendonça, Jansen-Alves, & Zambiazi, 2018; Ortiz Moreno, Dorantes, Galíndez, & Guzmán, 2003; Ramírez-Anaya, Manzano-Hernández, Tapia-Campos, Alarcón-Domínguez, & Castañeda-Saucedo, 2018; Werman & Neeman, 1987; Tan & Ghazali, 2019). There have also been multiple studies chemically characterizing avocado oil

based on cultivar (Fernandes et al., 2018; Manaf, Rahardjo, Yusof, Desa, & Nusantoro, 2018; Yanty, Marikkar, & Long, 2011) and region (Donetti & Terry, 2014; Tan, Tan, & Tan, 2017). However, there is a need to understand the range in quality and purity of the avocado oils currently on the market and how chemical composition of these oils compare to avocado oils characterized in literature. A few studies have done this on a small scale (Fernandes et al., 2018; Flores, Perez-Camino, and Troca, 2014; Werman & Neeman, 1987), however, to our knowledge no study has comprehensively evaluated the quality and purity of avocado oils available in the US, which is one of the largest consuming countries in the world (Altendorf, 2019).

Here we present an analysis of the quality and purity of avocado oils available in the US market with the goal of starting a database to support standards development for this industry. Twenty-two samples were collected from six grocery stores (14 samples) and two online sources (eight samples), efforts were made to cover all the major brands and types of oil (extra virgin/unrefined and refined). Oil quality was determined using free fatty acidity (FFA), peroxide value (PV), and specific extinction in ultraviolet (UV) absorbances in addition to chlorophyll and tocopherol content. The authenticity of the oils was assessed using the fatty acids, sterols, and triacylglycerols (TAG) profiles. This study aimed to better understand the quality and purity of avocado oils available in the US and to demonstrate that there is an urgent need for standards in this industry.

Materials and Methods

Avocado oil samples

A total of 22 avocado samples consisting of both extra virgin and refined oils were collected from six grocery stores (14 samples) and two online sources (eight samples). Each oil sample was wrapped in aluminum foil and stored in the dark at 20ºC. Samples were purged with

nitrogen after each opening. Table 2.1 contains information such as purchasing method, expiration date, product origin, cost and packaging type for each oil. Samples were separated into three groups according to their label. Extra virgin oil was coded as "EV" in front of the sample number, refined avocado oil as "R", and unspecified oils "U". The unspecified oils were samples that either did not specify the type of avocado oil or, samples that had unclear and ambiguous labels on the bottle.

Quality Parameters

FFA, PV, UV specific extinction at 232nm, 270nm, and ΔK were determined using AOCS methods Ca 5a-40 (09), Cd 8b-90 (09), and Ch 5-91 (09) (American Oil Chemist's Society, 1998), respectively.

Minor Components

Chlorophylls were determined according to AOCS method Cc 13d-55 (09) (American Oil Chemist's Society, 1998). Tocopherols were determined according to Gimeno et. al. (2000) with some modifications. Oil (40 µL) and hexane (160 µL) were briefly vortexed. The internal standard, α-tocopheryl acetate (purity 98%, Fisher Scientific Company LLC, USA) in ethanol at a concentration of 300 µg/mL, was then added in addition to 600 µL of methanol. The sample was vortexed for one min and centrifuged (5000 rpm, 5 min, Beckman GS-15R). Samples were stored at -20°C for two hours to allow oil to separate from the organic phase. The organic extract was filtered (0.45 µm, nylon). Analysis was performed on an Agilent 1290 Infinity II LC system with a diode-array detector using an Agilent ZORBAX Eclipse Plus C18 column (3.5 µm, 3 x 100 mm). The mobile phase was methanol:water (96:4), isocratic. A 20 µL injection volume and flow rate of 1.0 mL/min were used giving a total run time was 12 min. DAD signal was recorded

at 292 nm. All solvents used above were HPLC grade, from Fisher Scientific LLC, USA. Standards α-tocopherol (>96%), and α-tocopheryl acetate (98%) were purchased from Fisher Scientific LLC, USA. Analytical grade standards δ-tocopherol and γ-tocopherol were purchased from MilliporeSigma, USA.

Purity Parameters

The IOC official method for the determination of the fatty acid methyl esters by gas chromatography (COI/T.20/ Doc. No 33/Rev.1, 2017) was used for fatty acid profile analysis (International Olive Council, 2017). The GC-FID analysis was conducted on an Agilent 7890A GC (Agilent Technologies, USA). A 20 m × 180 μm × 0.20 μm DB-23 capillary column (Agilent Technologies, USA) was used to achieve the separation of individual fatty acids. The injection volume was 1.0 μL and helium, ultra-high purity, Airgas, USA was used as a carrier gas at a flow rate of 1 mL min^{-1}. The injector temperature was held at 250 °C at a split ratio of 50. The GC oven program was initially held at 80°C for 0.5 min; then ramped at 65°C min^{-1} to 175 °C, followed by a ramp of at 10°C min^{-1} to 185 °C, which was held for 0.5 min. The last ramp was at 7°C min^{-1} to 230°C and held for 5 min, giving a total run time of 14.89 min. The FID temperature was 260°C. The detector gas consisted of hydrogen, ultra-high purity, Praxair, USA (flow rate: 40 mL min^{-1}), air, specialty grade zero air, Praxair, USA, (flow rate: 400 mL min^{-1}), and helium, ultra-high purity, Airgas, USA make up gas (flow rate: 25 mL min^{-1}). Peak identification was performed using a FAME C8-C22, certified reference material, TraceCERT, MilliporeSigma, USA.

The IOC official method for the determination of the composition and content of sterols (COI/T.20/ Doc. No 30/Rev.1, 2013) was used with modifications (International Olive Council, 2013). The unsaponifiable fraction was prepared by drying 0.5 mL of internal standard 0.2% α-

cholestanol, analytical grade standard, MilliporeSigma, USA, ethyl acetate solution under nitrogen before adding 50 mL of 2 mol L^{-1} ethanolic potassium hydroxide, >85%, Fisher Scientific LLC, USA, to 5 g of the avocado oil sample. The mixture was heated to gentle boiling and kept under reflux for 20 min. The organic/aqueous mixture was extracted three times, 200 mL ethyl ether in total, washed with DI water, dried with anhydrous sodium sulfate, >99%, Fisher Scientific LLC, USA, evaporated to dryness, and further dried in an oven. The sterols were separated from the other unsaponifiable fractions on a silica gel $60F_{254}$-coated aluminum-backed thin-layer chromatography (TLC) sheet (MilliporeSigma, USA) with hexane/ethyl ether (60:40, v/v). The sterols band was made visible by spraying the plate with 0.2% 2, 7-dichlorofluorescein, ~90% (TLC), MilliporeSigma, USA, ethanolic solution and was then dissolved in 10 mL hot ethyl acetate and 30 mL ethyl ether and evaporated to dryness. All solvents used above were of HPLC grade from Fisher Scientific LLC, USA. Finally, 300 μL of the silylation reagent (pyridine, >99%, Fisher Scientific LLC, USA /hexamethyl disilazane, >99%, MilliporeSigma, USA /trimethylchlorosilane, >99%, MilliporeSigma, USA, 9:3:1, v/v/v) was added to prepare the trimethylsilyl ethers for GC injection. The GC-FID analysis was conducted on an Agilent 7890A GC (Agilent Technologies, USA). A 30 m × 0.25 mm × 0.25 μm DB-5 capillary column (Agilent Technologies, USA) was used with an injection volume of 1.0 μL and helium, ultra-high purity, Airgas, USA, as the carrier gas at a flow rate of 1.2 mL min^{-1}. The injector temperature was held at 280 °C at a split ratio of 25. The GC oven program was held isothermally at 150 °C for 8 min; then ramped at 20 °C min^{-1} to 290 °C and held for 20 min to obtain a total run time of 37.33 min. The FID temperature was 300 °C. The detector gas consisted of hydrogen, ultra-high purity, Praxair, USA (flow rate: 30 mL min^{-1}), air, specialty grade zero air, Praxair, USA (flow rate: 400 mL min^{-1}), and helium, ultra-high purity, Airgas,

USA, make up gas (flow rate: 25 mL min^{-1}). Peak identification was carried out with standards campesterol (65%), stigmasterol (95%), β-sitosterol (95%), each from MilliporeSigma, USA and by comparing the generated chromatograms against the sample chromatograms provided in the IOC official method and their relative retention times while the quantification was performed using the peak area and concentration of the internal standard.

TAGs were separated and analyzed using the method described in Green et al., (2020). In brief, each oil was diluted to a final concentrate of 1% with chloroform and then analyzed with the Vanquish™ Flex UHPLC-CAD system (Thermo Fisher Scientific, Waltham, MA, USA). Analytes were separated on a Thermo Scientific™ Accucore™ C18 column (100 mm x 2.1 mm; 2.6 µm). The injection volume was 1 µL and the flow rate was 0.5 mL/min. Mobile phase A was acetonitrile and mobile phase B was isopropanol using the solvent gradient conditions: start, 10% B; 2 min, 10% B; 25 min, 40% B; 30 min, 60% B; 35 min, 90% B; 40 min, 50% B and 45 min 10% B. All solvents were HPLC grade from Fisher Scientific LLC, USA.

Statistical analysis

Statistical analysis was accomplished using Originlab Corporation software version "OriginPro 2016 Sr2." This program was used to run PCA on all samples analyzed with the UHPLC-CAD. Principal component scores were computed by Originlab.

Results and Discussion

Quality parameters

Free fatty acids in the oil are caused by lipolysis where the fatty acids are separated from the TAG and are commonly used as a measurement for oil quality (CODEX, 2017; Woolf et al., 2009). The free fatty acid content of the oils is summarized in Figure 2.1a. Overall, samples

labeled as “extra virgin” had higher free fatty acidity than “refined” which is expected as the refining processes remove free fatty acids. The unspecified avocado oils had similar values to the refined, aside from U2 and U3, which had an FFA of 0.59% and 0.97%, respectively. Woolf et al. (2009) proposed the refined avocado oil should have values that are less than 0.1% FFA while Werman and Neman et al. (1987) saw about 0.55% FFA for refined oils (Werman & Neeman, 1987; Woolf et al., 2009). The refined oils in this study were all at or under 0.1%. Samples labeled as “extra virgin” had an FFA range of 0.03-2.69%, with an overall average of 1.31%. Commercial samples labeled as “virgin” analyzed in Flores et al. (2014) had FFA values ranging from 0.45-0.56%, while avocado oils made in-house in literature range from 0.12-2.84% (Bora, Narain, Rocha, & Queiroz Paulo, 2001; Krumreich et al., 2018; Manaf et al., 2018; Ortiz Moreno et al., 2003). The high values seen in this study could indicate use of poor-quality fruit and/or poor handling during processing, particularly for EV1, EV4 and EV5, which had values near 2.5%. Unhealthy fruits that are damaged, bruised, overripe, insect infested; prolonged time between harvest and processing; overheating during processing are all factors that can contribute to a rise in FFA (Woolf et al., 2009).

Peroxides are the primary oxidation products formed when an oil is exposed to oxygen and produce undesirable flavors and odors. The peroxide value results are shown in Figure 2.1b. Although trends within the three sample groups are less obvious than with the FFA results, overall, the refined oils had the lowest PV values averaging at 3.42 meq O_2/kg. The unspecified samples had a slightly higher average (4.13 meq O_2/kg); extra virgin samples were the highest at 7.4 meq O_2/kg. As with FFA, the refining process removes peroxides, therefore, lower values are expected for refined oils than those labeled extra virgin. However, many of the refined oils in this study still have notably high PV values. Woolf et al. (2009) proposed 0.5 meq O_2/kg to be

the upper limit for PV in refined avocado oils and standards proposed by Mexico for CODEX cap the acceptable PV at 2 meq O_2/kg. All of the oils except for R1, R3, and R5 were above these limits (CODEX, 2017; Woolf et al., 2009).

Table 2.1 contains sample information including purchasing method (in store or online), expiration date, product origin, cost and packaging type for each oil. Interestingly, the three refined oils with the highest PV values (R4, R8, and R9) were stored in clear instead of tinted packaging, which is not protective against photooxidation. Another factor that can contribute high PV values is storage time. The closer an oil is to the best by date on the bottle, the more likely it has had a long storage time. In this study, however, no correlation was found between the expiration date on the bottle and the PV values and all the samples were tested before reaching the expiration date. Literature values range from 1.4-12.74 meq O_2/kg for lab-made avocado oil samples (Jorge et al. 2015; Bora et al., 2001; Elez-Martinez et al., 2002; Krumreich et al., 2018; Manaf et al., 2018; Ortiz Moreno et al., 2003). A study looking at two commercial virgin avocado samples in Chile, storage time unknown, saw higher PV values of 8 meq O_2/kg and 12.95 meq O_2/kg (Flores, Perez-Camino, Troca, 2014). All of the samples tested in this study were in those ranges, aside from EV7, at 17.9 meq O_2/kg. Coincidentally, EV7 was the most expensive sample ($2.35/fl oz) out of the 22 samples purchased for this study.

K_{232} is another measure of the primary oxidation products present in an oil while K_{270} measures secondary oxidation products. Figure 2.2a shows the K_{232} values range from a low of 1.4 for sample R6 and EV1 to a high of 3.5 for sample U6. These ranges are comparable to values observed in the limited studies that have measured the specific extinction in UV in avocado oils. Ramirez-Anaya et al. (2018) saw K_{232} values of 1.8-2.8 for centrifuge extracted oil at different malaxation temperatures (Ramírez-Anaya et al., 2018). Another study looking at

commercial oils in Chile saw K_{232} values in the range of 3.16-4.19 (Flores, Perez-Camino, Troca, 2014). It is likely the increase of primary oxidation products seen in commercial samples from both this study and Flores et al. (2014) compared to the values seen in Ramirez-Anya et al. (2018) are because long storage time results in an increase of autoxidation.

Refined oils have a higher K_{270} because refining processes create conjugated trienes, which absorb at about 270 nm. Storage time can also increase K_{270} in avocado oils; Elez-Martinez et al. (2005) demonstrated that a fresh sample had a value of 0.4, which increased to 1.6 after 24 weeks (Elez-Martinez et al., 2002). In this study, the K_{270} was higher for many of the refined (average 0.725) and the unspecified oils (average 0.865) compared to the 0.459 average of the extra virgin samples. No correlation was seen between the expiration dates and K_{270} values. There was one unspecified oil, U4, with a particularly high K_{270} value of 1.84, which could indicate poor quality or harsh refining processes. When looking at the extra virgin samples EV3 and EV6 had higher K_{270} than the rest of the samples in this group. However, a K_{270} range of approximately 0.1-0.8 was seen in fresh in-house made oils under varying malaxation conditions (Ramírez-Anaya et al., 2018). This range was also seen in a study that analyzed commercial oils labeled as virgin (best-by date unknown), and is similar to the extra virgin oils in this study (0.16-0.77) (Flores, Perez-Camino, Troca, 2014).

The ΔK value can help distinguish virgin or extra virgin oil from one that is refined. The difference between a poor-quality virgin or extra virgin oil and one adulterated with refined oil can often be seen using ΔK (Vossen, 2007). Figure 2.2b summarizes the ΔK values for the oils in this study. To the best of our knowledge, this is the first report of ΔK values for avocado oil and we are therefore unable to compare values in this study with other literature. In the standards for olive oil from the International Olive Council, extra virgin olive oil must have a ΔK below 0.01

(Vossen, 2007). As anticipated, all of the refined oils are either at or above this limit as are all of the unspecified avocado oils with U1 having the lowest value of 0.01. U4 has gone under significant refining, with a value of nearly 0.2. For the extra virgin samples EV1, EV2, EV4, EV5, and EV7 are all under the extra virgin olive oil limit of 0.01. These are also the same samples that had a low K_{270}. This indicates it is likely that these oils are not adulterated with refined oils; however, some are of poor quality as they had high FFA and PV values. Interestingly, EV3 and EV6 which had low FFA and PV values and seemed to be the highest quality of the extra virgin samples had higher K_{270} and notably high ΔK values of 0.056 and 0.047, respectively compared to the other extra virgin samples. This indicates that it is possible that these two samples are refined or are blended with refined oils; the ΔK values for these two samples are still within the standard for refined olive oils, which must be below 0.16 (CODEX, 2017). In addition, the prices of these two samples were significantly lower than other extra virgin samples and more comparable with the refined oils.

Minor components

Chlorophyll pigments are what give extra virgin avocado oil its classic green color. In addition to the extra virgin labeled samples, three unspecified oils (U2, U3, and U6) were also tested as they appeared light green in color unlike the other refined oils and unspecified oils, which were light, pale yellow. The chlorophyll content ranged from 6.62 mg/kg to 98.8 mg/kg as shown in Figure 2.3. EV1, EV2, EV4, EV5, and EV7 contained ~95 mg/kg chlorophyll; these oils were noticeably dark green in appearance. The chlorophyll content seen in literature ranges greatly from 1.0 mg/kg to 69.8 mg/kg (Ashton et al., 2006; Jorge et al., 2015; Krumreich et al., 2018; Werman & Neeman, 1987; Wong et al., 2011). The inclusion of skin during processing could be responsible for the high values seen in this study. However, the values seen in Wong et

al. (2011) are lower than those seen in this study and in Ashton et al. 2006, which saw a chlorophyll content of to 214 mg/kg from the skin (Ashton et al., 2006; Wong et al., 2011). These variations are not unusual as the cultivar and ripeness of the fruit, extraction method, storage can all greatly impact the amount of chlorophyll in the oils. It's important to note that EV3 and EV6, which had the lowest chlorophyll content, were also the same oils that had low FFA and PV but high ΔK and K_{270}. This also supports the hypothesis that these oils are either refined or blended with oils that are refined.

There are eight compounds that make up vitamin E content, four tocopherols (α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol) and four tocotrienols. In this study, the four tocopherol compounds were quantified with beta and gamma values summed together (Table 2.2). Woolf et al. (2009) proposed that the tocopherol content in extra virgin avocado oil should be between 70-190 mg/kg (Woolf et al., 2009). Refined oils were not included in this range, as tocopherols are largely removed in the refining process. For all but three samples (EV3, EV6 and U6) in this study, alpha tocopherol was the highest concentration, followed by gamma, then delta which is consistent with literature (Fernandes et al., 2018; Madawalaa, Kochharb, & Duttaa, 2012; Manaf et al., 2018). However, the varietal can significantly impact the tocopherol content, for the Bacon avocado variety gamma tocopherol is higher than alpha (Fernandes et al., 2018). The lowest total tocopherol contents in this study were seen in R4 (34.0 mg/kg) and R9 (49.9 mg/kg). This study shows multiple samples (EV3, EV6, R1, U4, U5, U6) had total tocopherol contents over 400 mg/kg, which is interesting as the highest documented total tocopherol content in literature, to our knowledge, is 282 mg/kg (Corzzini et al., 2017). In particular, there are three samples with a notably high total tocopherol content, EV3, EV6 and U6 at 645.4 mg/kg, 906.2 mg/kg, and 692.9 mg/kg, respectively. These samples had significantly

higher levels of gamma and delta tocopherols compared to the other samples in this study and to values seen in literature for avocado oils. A study that reported on the tocopherol content in fruits and vegetables (Chun, Lee, Ye, Exler, & Eitenmiller, 2006), showed soybean oil has similar tocopherol levels and distributions to those seen in EV3, EV6 and U6, therefore, it is possible these samples contain soybean or had soybean tocopherols added after processing for preservation.

Purity parameters

Fatty acid profile is commonly used as a part of purity parameters to determine if an oil is adulterated. Table 2.3 shows the fatty acid profiles of all the samples which are consistent with literature with the exception of EV3, EV6 and U6. These three oils had linolenic acid (C18:3) values of 8.2-9.8%, while one of the highest values seen in literature was 3.19% in Hass variety (Tan et al., 2017). These oils also had a linoleic acid (C18:2) content of ~55%, substantially higher than seen in the other avocado oils in this study and from literature values, which were approximately 20% (Manaf et al., 2018; Tan et al., 2017). These oils also had high stearic acid (C18:0); low oleic (C18:1) and palmitic (C16:0) acids and their values for the fatty acid profile fit in the parameters for soybean oils from the CODEX standards for named vegetable oils (CODEX, 2017). The other oils in this study all had values comparable to literature with the exception of stearic acid (C18:0), which is higher in R1, R2, R3, R7, R8, U1, U4, and U5 than has been seen previously in literature (Berasategi, Barriuso, Ansorena, & Astiasarán, 2012; Bora et al., 2001; Fernandes et al., 2018; Forero-Doria, García, Vergara, & Guzman, 2017; Noorzyanna et al., 2017; Ortiz Moreno et al., 2003; Woolf et al., 2009). Samples R1, U4 and U5 also had lower palmitoleic acid (C16:1) compared to what has been reported in literature (Berasategi et al., 2012; Bora et al., 2001; Fernandes et al., 2018; Forero-Doria et al., 2017; Ortiz

Moreno et al., 2003; Ozdemir & Topuz, 2004; Tan et al., 2017). These deviations seen in the fatty acid profile could be a result of economic adulteration, however, due to lack of standards, one cannot easily make such claims. To support the establishment of standards, we need to build a database that includes natural variances such as climate, varietal, and growing region as these can impact the fatty acid profile of avocado oil.

The sterols profile is another purity parameter often used in conjunction with the fatty acid profile. Table 2.4 shows the sterols in all the samples. Samples EV3, EV6, and U6 had lower value of β-sitosterol of ~55% and higher values of campesterol and stigmasterol of ~20% and ~15%, respectively, which matched the sterols profile of soybean oil according to the CODEX standards. All other oils had values comparable to what has typically been seen in literature (Jorge et al., 2015; Fernandes et al., 2018; Madawalaa et al., 2012) with the exception of R1, U4 and U5. These oils are characterized by slightly higher amounts of campesterol, stigmasterol, Δ-7-stigmasetenol and Δ-7-avensterol and lower β-sitosterol. However, it has been shown avocado oil can have a β-sitosterol content as low as 73.9 mg/kg (Berasategi et al., 2012) and changes in extraction conditions can increase campesterol to values comparable to those seen in R1, U4, and U5 (Dos Santos et al., 2014). Like with the fatty acid profile results, it is necessary that a standard that accommodates natural variables such as cultivar, fruit maturity, irrigation and extraction methods. But this standard must also be able to discriminate pure avocado oil from an adulterated one in order to use sterols as a purity indicator for samples like R1, U4, and U5.

TAG profiles were determined for each oil and plotted using PCA as in Green et al. (2020) (Green et al., 2020). Figure 2.4 shows samples EV3, EV6, and U6 are located around the soybean oil cluster indicating they are likely 100% soybean oil and corroborating the fatty acid

and sterols profiles. All other avocado samples are in a separate group, close to the olive oils. This is expected as avocado, like olive oil, is high in TAGs containing oleic fatty acid and low in linoleic and linolenic. However, there are three samples R1, U4, and U5 are slightly removed from the other avocado oils in the cluster. These samples also have multiple values for their fatty acids and sterols profiles that are outside the range of 2xSD from pure samples in this study. This could be due to natural variance of the avocado fruits, processing conditions, or economic adulteration with high oleic sunflower or safflower oils. Preliminary analysis using the CODEX standards for vegetable oils suggested that 50:50 adulteration of avocado oil: high oleic sunflower could yield similar profiles as samples R1, U4, and U5.

Conclusions

This study demonstrates, for the first time, there are problems in both quality and purity in the store-bought extra virgin and refined avocado oil. The majority of the samples were of low quality with five of the seven oils labeled as “extra virgin” having high FFA values and six of the nine “refined” oils had high PV. FFA, PV, and specific extinction in UV data demonstrated that these oils have undergone lipolysis and oxidation, respectively. This likely resulted from improper or prolonged storage, using damaged or rotten fruits, or extreme and harsh processing conditions. Extra virgin oils often are more expensive and distinguished from lower grades such as virgin or crude oils using the above quality parameters.

Adulteration with soybean oil was found in two samples labeled as “extra virgin” avocado oil (EV3 and EV6) and one labeled as “pure” avocado oil (U6). Tocopherol, fatty acid, sterols, and TAGs data show this adulteration is occurring at or near 100% for all three samples. This not only is a potential health hazard for consumers but creates unfair competition in the market. EV3 and EV6 cost $0.65/fl oz and $0.49/fl oz, compared to the other extra virgin oils,

which averaged at $1.73/fl oz. Authentic extra virgin avocado oils are clearly being outcompeted by this economically motivated adulteration. In the case of samples EV3, EV6, and U6 the adulteration was confirmed in addition to the adulteration percent and adulterant oil. However, the need for standards is also demonstrated by the samples R1, U4, and U5. The variance seen in their fatty acid, sterols, TAGs, and tocopherols profiles could be due to natural variance of the avocado fruits, processing conditions, or unnaturally, economic adulteration with high oleic sunflower or safflower oils. In order to establish fair standards, it is also imperative to know how these parameters change with varietal, harvest time, and processing conditions to determine the appropriate ranges for avocado oil, ensuring authentic products are not flagged incorrectly. This study gives a timely overview of the quality and authenticity of the avocado oils available on the US market and a call to action for the standards establishment.

Competing Interests

The authors have no competing interests to declare.

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Tables

Table 2.1. Sample information for the oils used in this study.

Sample Code	Purchasing Method	Expiration Date (month-year)	Product Origin	Cost/fl oz ($)	Packaging Type
EV1	Online	Oct-21	California	2.23	Dark glass
EV2	In store	Jun-21	California	1.29	Dark glass
EV3	In store	Feb-21	Mexico	0.65	Dark glass
EV4	In store	Sep-20	California	1.53	Dark glass
EV5	Online	Jul-21	California	1.57	Dark glass
EV6	Online	NA	Brazil	0.49	Clear plastic
EV7	Online	Jun-21	California	2.35	Dark glass
R1	Online	Jun-21	Spain or Mexico	0.44	Dark plastic
R2	In store	Aug-20	Mexico	0.74	Dark glass
R3	In store	Nov-20	Mexico	0.43	Dark glass
R4	Online	Dec-20	Mexico	0.35	Clear plastic
R5	In store	May-20	Mexico	0.25	Dark plastic
R6	In store	Jul-20	Mexico	0.77	Dark glass
R7	Online	Dec-19	Mexico	0.80	Dark glass
R8	In store	Apr-21	Mexico	1.44	Clear glass
R9	In store	Apr-21	Mexico, USA, or Spain	0.29	Clear plastic
U1	In store	NA	Mexico	0.29	Dark plastic
U2	In store	Apr-21	Mexico, USA, or Spain	0.66	Tin bottle
U3	In store	Mar-21	Mexico, USA, or Spain	0.71	Tin bottle
U4	In store	May-21	Mexico	0.47	Dark glass
U5	In store	Jun-21	Mexico	0.79	Dark glass
U6	Online	Feb-21	Mexico	0.34	Clear plastic

Table 2.2. Individual and total tocopherol content, expressed in mg/kg, for each avocado oil (mean ± SEM, n=3). ND indicates tocopherol was not detected. Significant differences calculated using Tukey test.

	α-Tocopherol	**γ+β-Tocopherol**	**δ-Tocopherol**	**Total tocopherols**
EV1	155.2±11.8^{def}	ND	ND	155.2^{efghi}
EV2	116.0±4^{fgh}	ND	ND	116.0^{ghi}
EV3	87.3±3.2^{hi}	412.5±55.4^{b}	145.6±5.7^{c}	645.4^{b}
EV4	120.7±4^{fgh}	ND	ND	120.7^{ghi}
EV5	143.3±1.5^{efg}	ND	ND	143.3^{fghi}
EV6	95.9±0.5^{ghi}	581.3±67.1^{a}	229±9.7^{a}	906.2^{a}
EV7	140.9±11.9^{efg}	ND	ND	140.9^{fghi}
R1	396.7±8.7^{a}	108.8±4.7^{cd}	ND	505.5^{c}
R2	178.2±2.9^{cde}	ND	ND	178.2^{efgh}
R3	194.2±7.6 ^{cd}	102.6±21.3^{cd}	ND	296.8^{de}
R4	34.0±1.9^{j}	ND	ND	34.0^{i}
R5	116.9±2.6^{fgh}	ND	ND	116.9^{ghi}
R6	194.7±13.1^{cd}	ND	ND	194.7^{efg}
R7	209.3±12.2^{c}	ND	ND	209.3^{efg}
R8	276.8±15.3^{b}	ND	ND	276.8^{ef}
R9	49.9±6.7^{ij}	ND	ND	49.9^{hi}
U1	156.8±6.2^{def}	ND	ND	156.8^{efghi}
U2	52.2±0.2^{ij}	42.4±1.4^{cd}	ND	94.6^{ghi}
U3	60.1±9.3^{ij}	41.5±5.4^{cd}	ND	101.6^{ghi}
U4	317.6±20.9^{b}	106.8±5.3^{cd}	ND	424.4^{cd}
U5	388.0±12.7^{a}	129±5.2^{c}	ND	517.0^{c}
U6	91.1±0.2^{hi}	440±48.1^{b}	161.8±6.6^{b}	692.9^{b}

Table 2.3. Fatty acid profile expressed in percent of total fatty acids for each avocado oil (mean ± SEM, n=2). ND indicates fatty acid was not detected.

	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0	C24:0
EV1	ND	16.5±0.1	6.9±0	0.5±0	55.6±0.1	19.2±0.1	1.2±0	ND	0.1±0.1	ND	ND
EV2	0.1±0	15.6±0	6.5±0	0.5±0	61.0±0	15.2±0	1.0±0	ND	0.2±0	ND	ND
EV3	0.1±0	10.9±0	0.1±0	4.0±0	21.4±0.1	54.4±0.1	8.2±0	0.3±0	0.2±0	0.3±0	0.1±0
EV4	0.1±0	15.5±0	6.4±0	0.5±0	59.3±0.1	17.0±0.1	1.1±0	ND	0.2±0	ND	ND
EV5	0.1±0	15.6±0	6.4±0	0.5±0	58.6±0	17.5±0	1.1±0	ND	0.2±0	ND	ND
EV6	0.1±0	10.4±0	0.1±0	3.8±0	19.7±0.5	55.4±0.4	9.8±0	0.4±0	0.2±0	0.3±0	0.1±0
EV7	ND	16.0±0	6.6±0	0.5±0	62.4±0	13.4±0	0.9±0	ND	0.2±0	ND	ND
R1	ND	10.0±0	1.7±0	2.3±0	69.1±0	15.2±0	0.5±0	0.3±0	0.3±0	0.4±0	0.2±0
R2	ND	14.7±0	5.8±0	1.4±0	64.4±0.1	12.2±0	0.7±0	0.2±0	0.3±0	0.2±0	0.1±0
R3	ND	13.2±0	4.2±0	1.4±0	63.8±0.1	16.0±0.1	0.7±0	0.2±0	0.3±0	0.2±0	0.1±0
R4	ND	15.8±0	6.8±0	0.5±0	63.8±0	12.0±0	0.8±0	ND	0.2±0	ND	ND
R5	ND	15.0±0	6.5±0	0.8±0	63.6±0	12.8±0	0.8±0	0.1±0	0.2±0	0.1±0	ND
R6	ND	17.8±0	8.6±0	0.6±0	61.0±0.1	10.9±0	0.8±0	0.1±0	0.2±0	ND	ND
R7	ND	14.4±0	5.2±0	1.4±0	64.8±0	13.0±0	0.7±0	0.2±0	0.2±0	0.2±0	0.1±0
R8	ND	13.4±0	5.1±0	1.6±0	67.5±0	10.9±0	0.6±0	0.2±0	0.2±0	0.3±0	0.1±0
R9	ND	14.1±0	5.2±0	1.0±0	63.2±0	15.0±0	0.8±0	0.2±0	0.2±0	0.1±0	0.1±0
U1	ND	16.5±0	7.4±0	1.3±0	63.9±0	9.8±0	0.7±0	0.2±0	0.2±0	ND	ND
U2	ND	16.4±0	7.2±0	0.6±0	60.0±0	14.7±0	0.9±0	ND	0.2±0	ND	ND
U3	ND	16.5±0	7.4±0	0.6±0	60.4±0	13.9±0	0.8±0	0.1±0	0.2±0	ND	ND
U4	ND	10.4±0	2.0±0	2.1±0	66.5±0	17.4±0	0.5±0	0.4±0	0.3±0	0.2±0	0.1±0
U5	0.1±0	11.2±0	0.6±0	2.8±0	68.3±0	15.4±0	0.5±0	0.4±0	0.3±0	0.4±0	0.2±0
U6	0.1±0	10.9±0	0.1±0	4.0±0	21.0±0	54.7±0	8.2±0	0.3±0	0.2±0	0.3±0	0.1±0

Table 2.4. Sterols profile for each avocado oil as mean (%) ± SEM, n=2. Total sterols is expressed in mg/kg. ND indicates sterol was not detected. Each sterol is abbreviated and ends in -sterol, except for Δ7-stigmastenol.

	Bras-sica	Campe	Stigma	Δ7-campe	Clero	β-sito	Δ5-Avena	Δ7-Stigma	Δ7-Avena	Total sterols (mg/kg)
EV1	0.4± 0.4	5.5±0	0.8±0.2	ND	1.9±0.1	85.6±0.5	5.7±0.3	ND	ND	5955±110
EV2	ND	5.4±0.3	ND	ND	1.9±0.1	86.8±0.7	5.8±0.3	ND	ND	4670±200
EV3	ND	20.3±0.1	15.8±0.1	ND	ND	56.3±0.1	2.7±0	2.8±0.2	2.1±0.3	2601±75
EV4	ND	5.6±0.1	0.6±0	ND	1.8±0	86±0.3	6.0±0.3	ND	ND	5649±200
EV5	ND	5.8±0	0.6±0	ND	1.9±0	85.4±0.3	6.3±0.3	ND	ND	5245±140
EV6	ND	23.3±0.1	15±0.2	ND	ND	55.2±0.1	3.8±0.2	1.5±0.1	1.3±0.1	3306±0
EV7	ND	6.3±0	ND	ND	1.9±0	86.3±0.1	5.6±0.1	ND	ND	4263±31
R1	ND	8.6±0.2	4.6±0.1	ND	0.9±0	75.6±0.2	4.5±0.2	4.3±0.1	1.4±0.2	2906±10
R2	ND	5.7±0	1.4±0	ND	1.2±0	85.7±0.1	4.6±0.1	1.5±0.1	ND	3356±48
R3	ND	7.6±0.3	2.2±0.2	ND	1.3±0.1	81.4±1.9	5.2±0	2.2±2.2	ND	3362±56
R4	ND	4.9±0	0.4±0	ND	1.4±0	87.1±0	5.6±0.1	ND	ND	3850±3.0
R5	ND	5.6±0	0.9±0	ND	1.3±0	86.0±0	5.2±0	0.5±0	ND	3926±14
R6	ND	6.3±0	0.6±0	ND	1.5±0	86.5±0.1	5.1±0.1	ND	ND	3553±25
R7	ND	5.8±0	1.3±0	ND	1.2±0	87±0.1	4.8±0.1	ND	ND	3344±74
R8	ND	6.1±0.1	2.5±0	ND	1.3±0.1	81.1±0.3	4.6±0.2	3.4±0	1.2±0.1	3168±170
R9	ND	9.1±0	2.1±0	ND	1.4±0	81.4±0.1	5.9±0.1	ND	ND	4125±73
U1	0.4± 0.4	6.0±0	0.6±0.2	ND	1.2±0	88.4±0.4	3.5±0.2	ND	ND	2859±70
U2	ND	7.7±0.6	1.1±0	ND	1.6±0	83.5±1.1	6.0±0.5	ND	ND	4066±250
U3	ND	6.8±0.2	1.1±0	ND	1.6±0	84.9±0	5.6±0.2	ND	ND	4340±69
U4	ND	10.1±0.1	3.8±0.1	0.7±0	0.9±0.1	74.7±0	4.6±0.2	4.0±0.2	1.2±0.1	3341±95
U5	ND	9.2±0.1	4.8±0.1	ND	ND	77±0.2	3.6±0.1	4.2±0.2	1.2±0.1	3465±66
U6	ND	20.6±0.2	16.2±0.4	ND	ND	56±0.4	2.5±0.3	2.8±0.3	1.8±0.1	2678±130

Figures

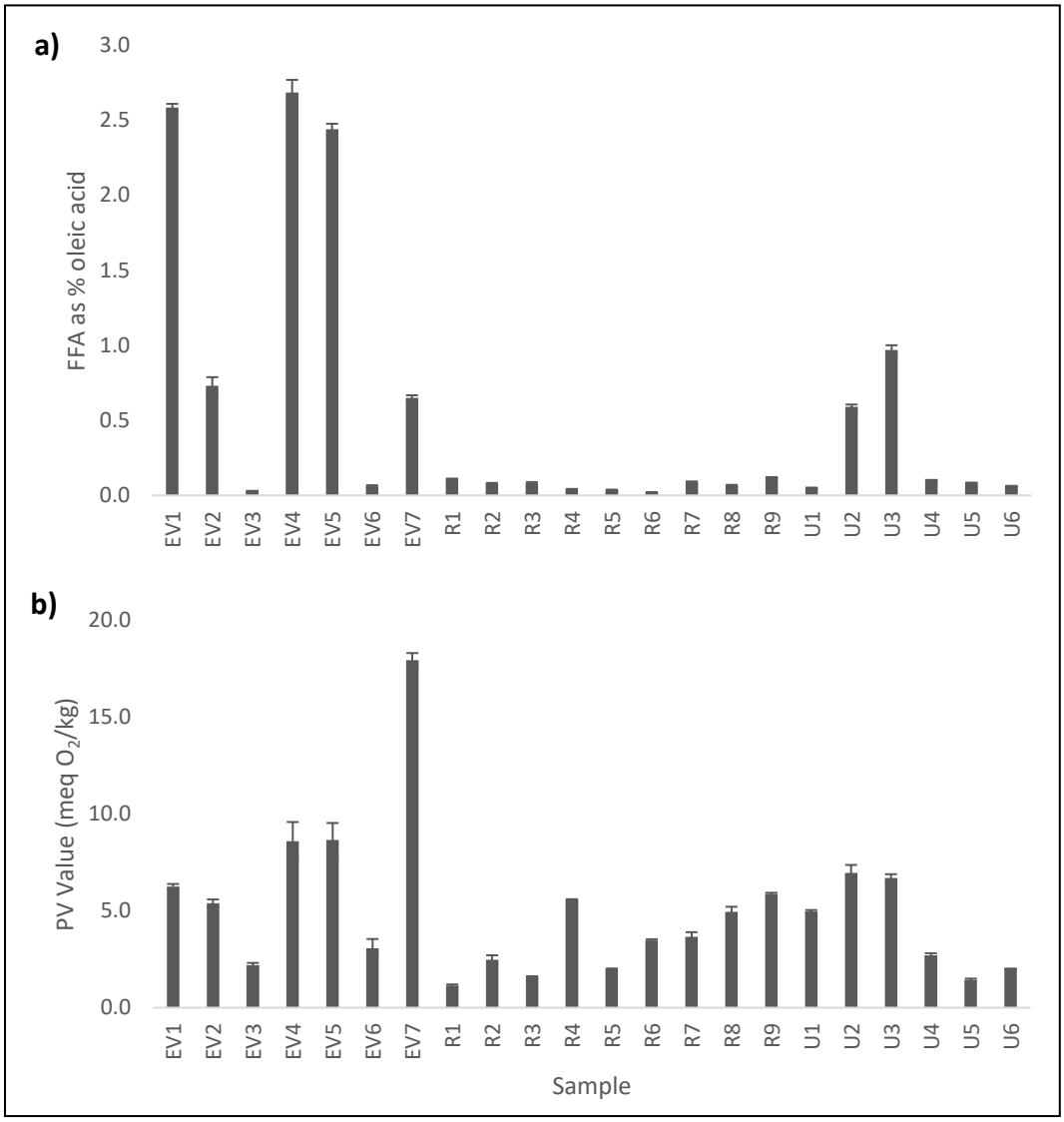

Figure 2.1. (a) Free fatty acid content reported as % oleic fatty acid. (b) Peroxide value expressed in meq O_2/kg. Each bar is an average of triplicate measurements and error bars are calculated using the standard error of the mean (SEM). EV stands for extra virgin, R for refined, and U for unspecified avocado oil.

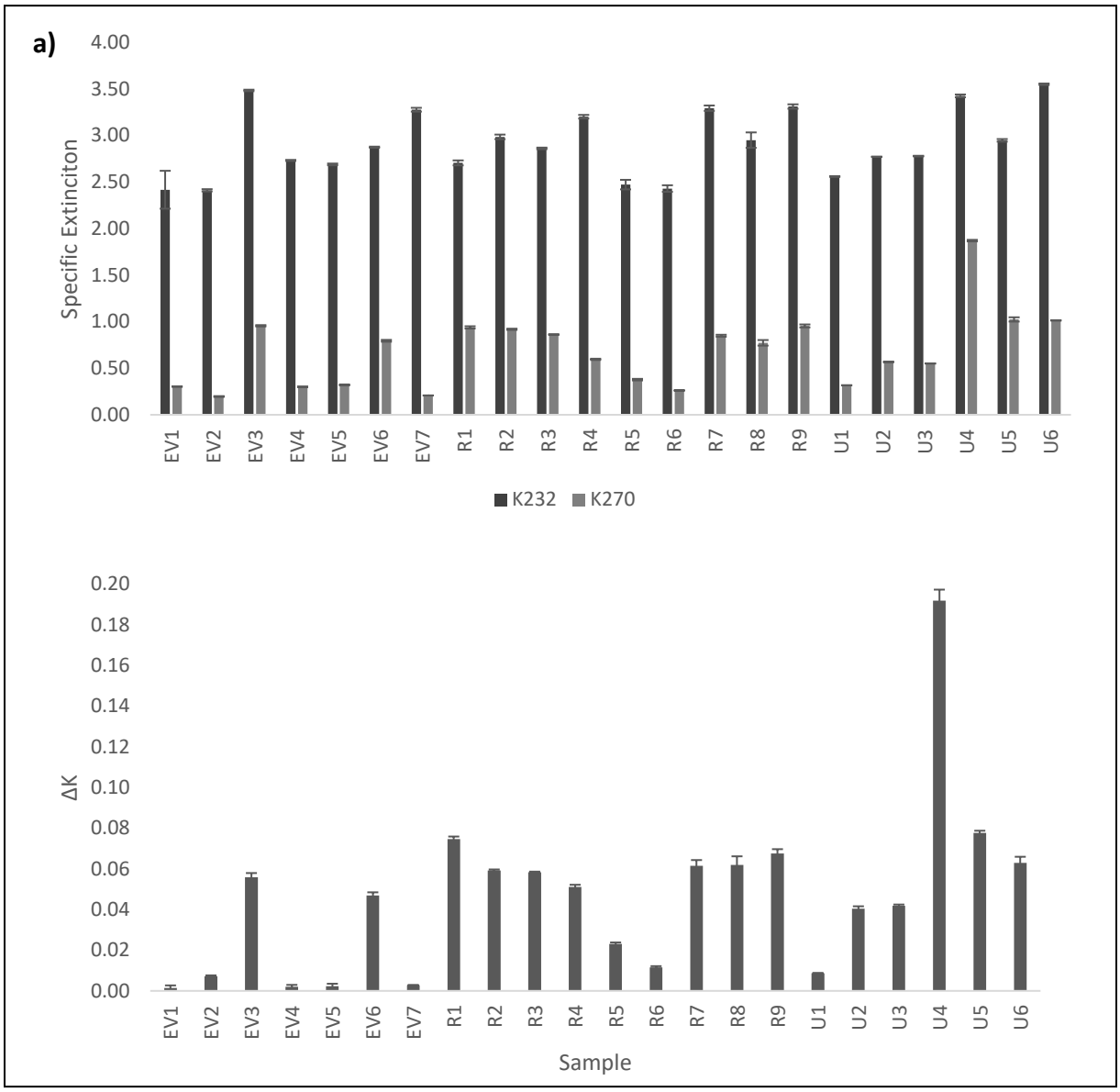

Figure 2.2. (a) Values for the primary oxidation products (K_{232}) and secondary oxidation products (K_{270}) in each oil. (b) Values for ΔK. Bars are an average of triplicate measurements and error bars show SEM. EV stands for extra virgin, R for refined, and U for unspecified avocado oil.

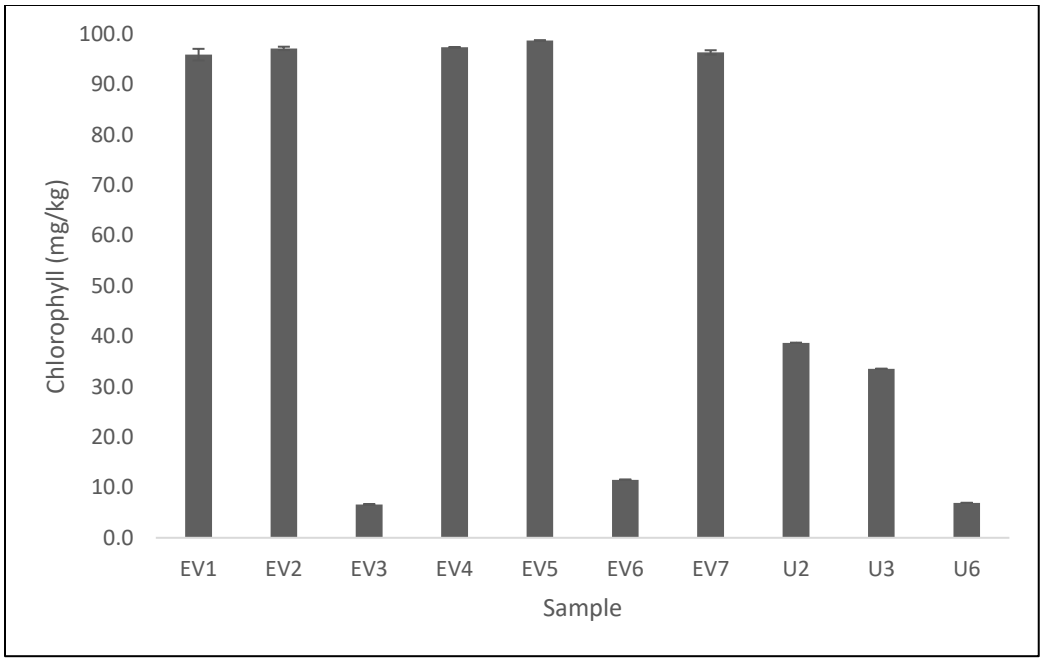

Figure 2.3. Total chlorophyll content determined by AOCS official method Cc 13d-55. Measurements are done in triplicate with error bars indicating SEM. EV stands for extra virgin, R for refined, and U for unspecified avocado oil.

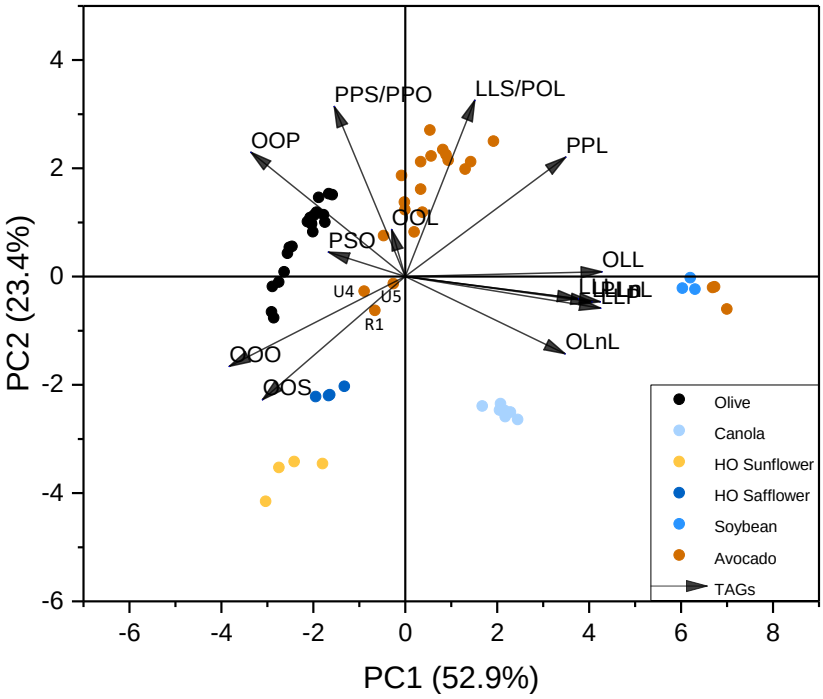

Figure 2.4. TAG profiles plotted using PCA. The six avocado oils that differed from other samples are labeled according to their sample codes. All other avocado oils from this study are labeled as avocado, shown in dark orange.

Chapter 3

Extra virgin grade avocado oil can be achieved using whole fruits or only mesocarp

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Abstract

Avocado fruits were collected throughout the season from California, USA and Michoacan, Mexico. Oil was made from high quality, Grade 1, and low quality, Grade 4, avocados from both regions using a laboratory-scale oil extraction mill through physical means. For each grade, oil was made from whole fruits and only the mesocarp. The impact of each of these parameters on free fatty acidity, peroxide value, specific extinction coefficients at 232 and 270 nm (K_{232} and K_{270}), and the total phenolic content was determined. Results showed that fruit quality grade had the biggest effect on free fatty acidity; peroxide value was largely unaffected; and both grade and processing using whole versus mesocarp effected specific extinction coefficients values. Oil made from Grade 4 avocados had a higher total phenol content than Grade 1, with whole fruit having higher values overall than mesocarp. This is the first study that suggests avocado oil mechanically extracted from whole fruits can meet the grade standard for virgin and/or extra

virgin. The ranges for each of the above quality parameters from this work can serve for standard establishment purposes.

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Background

Avocado oil is becoming an increasingly popular product and ingredient. This, combined with our recent study on the quality and purity of commercial brands of avocado oil, has motivated industry members and regulators to work together on establishing standard for this product (Green and Wang, 2020; CODEX, 2021b). An important part of the standard development process is to include input and research from traditional and new production countries, which for avocado oil include Mexico, South Arica, Kenya, Brazil, Peru, New Zealand, and the United States. However, an ongoing challenge is that the extent in which how natural variables impact avocado oil are not well-documented or well-understood especially for newer producing regions. To develop appropriate standards, it is necessary to analyze the chemical composition of avocado oil according to variables such as growing region, harvest time, maturity, ripeness, and processing. Although understanding these impacts is needed for both quality and purity criteria, this study focuses on the quality parameters.

Currently, refined avocado oil dominates the market in the United States, which has the benefit of high monounsaturated fatty acids and phytosterols (Fernandes et al. 2018, Wang et al. 2019, Wong et al. 2010). However, there are additional health benefits specifically associated with unrefined virgin/extra virgin oils – made solely through mechanical means from healthy fruits without heat or solvents. The most common unrefined oil is virgin/extra virgin olive oil containing higher concentrations of antioxidants like phenolics than their refined counterparts. Interest in unrefined avocado oil is rising and extra virgin grade already makes up a majority of the avocado oil market in New Zealand (Woolf et al. 2009). Because virgin/extra virgin oils are not processed with solvent nor go through any of the refining processes (bleaching, deodorizing,

winterizing) that remove compounds such as free fatty acids, virgin/extra virgin oils require an independent set of quality standards from refined or crude oils.

A large portion of literature on avocado oil has been on optimizing extraction conditions, as the yield avocado oil is known to be poor. Although extra virgin processing of avocado oil has been done (Werman and Neeman, 1987; Ramirez-Anaya et al. 2018; Martinez-Padilla et al. 2018; Wong et al. 2014), many of these methods rely on heating, drying in ovens or microwave (Dos Santos et al. 2014; Moreno et al. 2003), solvent extraction (Corzzini et al. 2017; Dos Santos et al. 2014; Mostert et al. 2007) or newer, unconventional extraction methods such as ultrasound assisted extraction (Tan et al. 2018). To develop appropriate quality standards for unrefined avocado oil, it is important to not only evaluate how quality parameters can be impacted by natural variables but also simulate mechanical extraction processing in the laboratory.

Here we present an analysis of the quality of avocado oils made via a laboratory extraction mill that is similar to the industry uses for cold-press processing. Two different levels of quality fruits were used because the olive oil industry has shown that bad quality fruit can lead to bad quality oil. A key difference between olive oil and avocado oil is that olives are not commonly sold as fresh fruits due to natural bitterness whereas fresh avocados are in high demand and cherished by the consumers worldwide. Using both quality levels will demonstrate the range of chemical quality parameters for avocado oil and what reasonable parameters of a pure extra virgin oil should be. Most of the work on extra virgin avocado oil so far has been completed in New Zealand (Woolf et al. 2009; Wong et al. 2011; Requejo-Tapia et al; Wong et al. 2014; Ashton et al. 2006; Yang et al. 2018; Yang et al. 2020), therefore, for this study, we focused on fruits from California and Mexico, harvested over the course their respective harvest seasons. Studies from various olive oil producing countries have shown that harvest time and

olive fruit maturity can impact quality parameters such as free fatty acidity and peroxide value (Yousfi et al. 2006; Dag et al. 2011; Qarnifa et al. 2019; Gutierrez et al. 1999; Polari et al. 2020 and 2021). Two types of oils were made for each extraction, oil made from the whole fruit (as how olive oil is made) and oil made from only mesocarp. This is motivated by an inquiry if virgin/extra virgin grade can also be made from whole fruit and/or if different standards are need for oil made from the whole fruit versus mesocarp, a topic currently relevant to industry members and regulators for standards development. Oil quality was determined based on free fatty acidity (FFA), peroxide value (PV), and the coefficients of specific extinction in ultraviolet (UV) absorbances in addition to total phenolic content (TPC). This is the first study to show if virgin/extra virgin avocado oil can be made from whole fruit, which could greatly expand the high quality unrefined avocado oil industry, currently dominated by refined oils. By being able to use the whole fruit in oil processing there is also the potential to reduce food waste, where the pit and skins are used instead of discarded.

Materials and Methods

Experimental Design

Hass avocados were collected from two different regions, California, USA (CA) and Michoacán state in Mexico (MX) throughout each location's harvest season during the year of 2021. There were three sampling periods for CA fruit, which occurred in March, June, and August and five for MX fruit: February, April, June, August, and November. For each timepoint fruit was collected from three different growers lots for both CA and MX locations. Two grades of fruit were processed into oil from each region/harvest time: Grade 1 (G1) and Grade 4 (G4), images of each grade shown in Figure S3.1. In brief, G1 are of high-quality fruits at eating ripeness while G4 are of low-quality fruits with extreme rotting. Fruits were stored at 20°C and

80-90% humidity with apples as an ethylene source until they reached the appropriate ripeness (typically one week for G1 and 2-2.5 weeks for G4) and were then processed immediately. G1 and G4 fruits were split in half and processed two ways to make oil, either using the whole fruit (exocarp/skin, seed/pit, and mesocarp/flesh) to or only the mesocarp/flesh.

Avocado oil extraction

A laboratory-scale oil mill Abencore system (MC2 Ingenieria y Sistemas S.L., Seville, Spain) was used to extract oil from avocados. Fruit (either whole fruit or only mesocarp) were crushed into a paste with a Schneider Electric (GV2 MC01) crusher. Once crushed, 700g of avocado paste was malaxed with 10% weight (70g) talc, 3 mL of enzyme (Novozyme Pectinex Ultra Olio) for one hour at 50°C. Halfway through, 140 mL of water was added (or 20% of the mass of the avocado paste used). After malaxation, the paste mixture was centrifuged for 3-4min in a basket centrifuge. Oil was decanted and then re-centrifuged at 10,000 rpm for 10min. Plastic bottles were filled to the top with oil and stored in a dark freezer at -20°C until chemical analysis.

Moisture and fat content

Avocado paste (60 ± 0.1 g) was weighed in an aluminum dish (Fisher Scientific) and placed in the oven at 105°C for 12 h or until all water had evaporated. The dish was removed from the oven, cooled, then weighed to determine moisture content. The dried sample was then used to determine fat content. Dried samples were ground using a food processor and weighed (10 ± 0.1 g) into a cellulose thimble (33x150mm, Whatman 603 Cellulose thimbles). Samples were extracted using the Buchi Universal Extractor E-800using *n*-hexane. Once the extraction finished, remaining residual solvent was evaporated in the fume hood and fat content was

calculated (in both wet and dry basis) using mass of oil collected from extraction. Extraction efficiency was calculated using oil yield and fat content in wet basis.

Quality Parameters

Free fatty acidity (FFA), peroxide value (PV), and the specific extinction coefficients in UV (K_{232}, K_{270}, and ΔK) were determined using AOCS methods Ca 5a-40 (09), Cd 8b-90 (09), and Ch 5-91 (09), respectively.

Total phenolic content

Total phenols were determined using the method described in Bajoub et al. 2015 with several modifications to accommodate the low phenolic content observed in these avocado oils. Oil (2.0 ± 0.1 g) was dissolved in 1 mL hexane and extracted twice with 1mL of methanol:water (60:40, v/v). Samples were centrifuged at 4000rpm for 10 min after each extraction, then supernatants were combined. A 0.5 mL aliquot was taken from the combined extract and diluted with water to 5 mL, followed by the addition of 0.5 mL Folin-Ciocalteu reagent and 1mL of 30% w/v sodium carbonate. The solution was mixed and stored in the dark for 24 hours, after which the absorbance was measured at 725 nm. An external calibration curve was made using, and expressed as, gallic acid.

Statistical analysis

Statistical analysis was performed using a three-way ANOVA and was visualized using ggplot 2 in R. Statistical significance was assessed separately for each region, where p-values were used to determine significance and f-statistic, effect size.

Results and Discussion

Extraction variables

Extraction efficiency along with the moisture and fat content are shown in Figures 3.1a and b. There was a general trend of increasing extraction efficiency throughout the season for both regions, although this was not the case for every timepoint comparison (ex. Figure 3.1a early and early/mid season MX samples). There was also a trend of increasing fat content throughout the season, however, due to high variance in the fat content this is less clear. In both Figure 3.1a and 1b MX samples show there was often an inverse relationship between moisture and fat content. It has been well-documented in literature that as moisture content decreases throughout the season fat content increases (Kikuta and Erickson, 1968; Pearson, 1975), which can improve extraction efficiency.

Woolf et al. 2009 notes that fat content doesn't change after avocado has been harvested from the tree, which was consistent with this data as there was not a significant difference in fat between G1 and G4 fruits. Figure 3.1b compares the parts of the fruit used in extraction instead of fruit grade in Figure 3.1a. The mesocarp had slightly higher moisture content, and higher fat content compared to whole fruit. Skin and pit contribute to volume of paste in the extraction but have less moisture than the pulp and can contain minimal fat content (Galvao et al. 2014).

The oil often did not separate as well for the mesocarp-only extractions, particularly for G1 fruit. For these samples, two different centrifugations were necessary to ensure all oil was collected. The first was a basket centrifuge used to separate the oil from the water and fruit solids. After this step the oil layer was centrifuged again in a fixed-rotor centrifuge at a higher RPM and longer time to further purify the oil. Overall, the second centrifugation had minimal impact on the yield and extraction efficiency, however, it may need to be considered depending

on the type of extraction equipment and processing procedure being used. This was likely less of a problem for whole fruit extractions because the skin and pit help to break the cell walls for oil release as well as interrupt emulsion allowing for better separation of oil. Because both grade of fruit and the parts of the fruit used in oil extraction can impact on the ease of extraction, further studies are needed to explore these relationships to help processors maximize yield without impacting the quality of their oil.

Oil hydrolysis: Free fatty acidity

Free fatty acids are an indicator of oil quality and limits are often included in grade standards (CODEX, 2021a; IOC, 2019; USDA, 2010; CDFA 2021). A high FFA value indicates the fruits may have been damaged and/or fermented and the oil is of poorer quality, as more fatty acids have separated from the triacylglycerol molecule (TAG) via hydrolysis (Woolf et al., 2009). Figure 3.2a and 3.2c show that grade had the biggest impact on FFA for both CA and MX samples (p-value=$2.5x10^{-9}$ for CA and $4.6x10^{-7}$ for MX), which was supported by the large effect size for grade (f-statistic 73 for CA and 33 for MX compared to 5 or less for all other variables), calculated using ANOVA. Oils made from G1 fruits had significantly lower FFA than oils made from G4 within each region, shown in Figure 3.2c. This is expected, as more free fatty acids are formed in unhealthy, bruised, insect infested, and overripe fruits (Woolf, 2009). The G4 fruits used in this study had many of these characteristics (image shown in Figure S3.1). There was one set of grade 4 fruits, mid season MX, that had less bruising and physical damage compared to the other G4 fruits used in this study. The oils made from these fruits had lower FFA than the other G4 samples. This is a further example for how strongly correlated FFA is with unhealthy, bruised, and damaged fruit. The scale in Figure 3.2b helps to show differences among smaller FFA values where it can also be seen that whole fruit had higher FFA content than mesocarp for

CA, however this trend is absent for MX. Few, if any studies have been done comparing the oil from the mesocarp of the avocado to the whole, however, Wong et al. 2011 compared how the addition of skin can impact oil quality. They found that there was not a significant effect on FFA when adding skin.

Table 3.1 shows the FFA values for G1, which ranged from 0.14 to 0.31 % as oleic acid and G4 from 0.41 to 8.0 %. Currently the proposed avocado oil standards by CODEX Alimentarius International Food Standards (CODEX), which was established by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), do not include virgin/extra virgin as a grade category, therefore their corresponding FFA limits are lacking (CODEX, 2021b). Researchers in New Zealand, which was the first country with a large extra virgin avocado oil market, proposed that FFA should be less than 0.5% as oleic acid for extra virgin oils and 0.8-1.0% for virgin oils (Woolf et al. 2009). The G1 samples in this study met the stricter, extra virgin limit. The G4 samples were significantly above this (as high as 8.0%) and would need to be refined to be consumed. For oils extracted via similar cold-pressed methods used in this study comparable FFA values were reported. Roda et al. 2019 made oils with a wider range of FFAs from 0.32-1.22%, however most samples were around 0.4%. Slightly lower ranges of 0.2-0.3% were also seen in Martinez Padilla et al. 2018 and 0.09-0.13% for Woolf et al. 2009.

Oil oxidation

Peroxide value

Peroxide value (PV) is a measure of the primary oxidation products, namely hydroperoxides, formed in an oil due to photooxidation, autooxidation, or oxygen exposure. PV

is a crude measurement of early oil oxidation and is often included in oil standards, similar to FFA. Figure 3.3 summarizes the PV values from the oils in this study. Unlike FFA, there was not an obvious trend with fruit grade, or any other variable used in this study (Figure 3.3a and 3.3b). When looking at only the CA plot in Figure 3.3b, there was a significant difference between G1 (lower PV) and G4 (higher PV) mesocarp samples, which was supported by a three-way ANOVA that showed grade contributed to significant difference for CA fruits (p-value= $1.6x10^{-5}$). However, for the MX fruit timepoint was the only parameter that contributed to significant differences between samples (p-value=$2.1x10^{-5}$). A stronger trend between grade and PV may also be lacking because PV only measures primary oxidation products, if these are then further oxidized or broken down then they will not be accounted for in this test; PV values follow a curve where they increase and then go back down as the hydroperoxides break down to secondary oxidation products (Frankel, 2005).

Figure 3.3b shows that in some cases there were significant differences between whole fruit and mesocarp samples (ex. MX, early season, G1 samples or between CA, early season, G1 samples). However, when comparing samples only according to their whole fruit/mesocarp labels, ANOVA demonstrated this variable was not responsible for significant differences between samples (p-value=0.21 for CA and 0.44 for MX). Oil using the whole fruit contains more chlorophyll, which is a known photosensitizer. Although oils were stored in a dark freezer until analysis, malaxation and processing were done using lab-scale equipment that was not in a closed system protected from light and oxygen, as is typical on industrial scale extraction. It was expected PV might increase under these conditions, but our results are consistent with Wong et al. 2011, who also found that extra skin in oil, which is the main source of chlorophyll in whole fruit oil, did not increase PV appreciably in oils. However, the specific extinction value K_{232},

discussed in section 3.3.2 tells a different story on how chlorophyll could be involved in oxidation.

The range of PV values seen in this study were 1.91-8.13 meq O_2/kg and 2.17-7.24 meq O_2/kg for G4 and G1 oils, respectively (Table 3.1). Literature ranges are quite broad for PV, with many sources seeing a much lower range from 0.8-3 meq O_2/kg (Woolf et al. 2009, Tan et al. 2018, Roda et al. 2019) while other sources report 12-20 meq O_2/kg as fruit and processing conditions varied (Martinez-Padilla et al. 2018 and Lopez-Vega et al. 2021). Eight of the G1 samples from this study did not meet Woolf's proposed limit of 4.0 meqO_2/kg for extra virgin oils (Woolf et al. 2009), likely because our laboratory oil extraction mill was an open system, which did not protect samples from oxygen exposure during malaxation like most industrial-scale equipment does. However, all samples met the virgin avocado oil limit of 8.0 meqO_2/kg. A limit of 4.0 meqO_2/kg for extra virgin and 8.0 meqO_2/kg for virgin may be too low avocado oils, as the oil should be able to meet this limit not only at the point of production but at the end of shelf life. The PV limit for virgin/extra virgin olive oil is 20 meqO_2/kg for the International Olive Council standards (IOC), and the United States Department of Agriculture (USDA) (CODEX, 2021a; IOC, 2019; USDA, 2010). The limit for extra virgin is lower at 15 meqO_2/kg for oils regulated by the State of California, Department of Food and Agriculture (CDFA, 2021), however, their limit for virgin oils is also 20 meqO_2/kg. If avocado oil were to adopt either of these limits, all of our G1 samples, regardless if they were made with whole fruit or mesocarp would qualify as extra virgin oils. More research is needed to determine if a value that is between 4 and 20 meqO_2/kg would be more realistic and achievable for virgin/extra virgin avocado oil produced around the world.

Specific Extinction

K_{232} is another measure of the primary oxidation products and can be considered more reliable than PV because a spectrophotometer is used instead of a titration done by eye and can detect a broader range of primary oxidation products than PV. K_{232} is not used as widely as PV and thus is not a part of current avocado oil proposed CODEX standards. K_{232} must be less than or equal to 2.5 for extra virgin oils (IOC, 2019; USDA, 2010) and 2.6 for virgin (IOC, 2019; USDA, 2010; CDFA, 2021). The G1 samples in this study ranged from 1.63-2.71, with many being around the 2.5-2.6 limits. Unlike PV, both grade and processing variables contributed to significant differences between samples, which can be seen visually in Figure 3.4b (p-value=$4.2x10^{-7}$ and $2.9x10^{-16}$ for CA and MX processing variable, respectively and p-value=$3.5x10^{-7}$and 0.0047 for CA and MX grade, respectively). Grade had a much larger effect size for the CA samples (f-statistic=43 than MX (f-statistic=8), which was consistent with the PV data, but the effect of processing was distinct from PV data. Figure 3.4a shows that K_{232} was always higher for whole fruit in MX and was higher the majority of the time in CA. It is likely there were oxidation products present in the whole fruit oils that were able to be detected with K_{232} and not with PV. Because chlorophyll is a photosensitizer it could be aiding in the formation of a greater number of oxidation products in the whole fruit compared to the mesocarp. There have been contradicting studies on the relationship between these measurements. Some have shown a correlation between the PV and K_{232} (Grigoriadou and Tsimidou et al. 2006) while others have shown the relationship is complex (Gutierrez et al. 1999, Katsoyannos et al. 2015, Polari et al. 2019 and 2020, and Yousfi et al. 2006). No correlation (R^2=0.0631) was observed for our samples. Oils from each timepoint were also significantly

different within CA and MX according to K_{232}, however, this is more likely due to variation in the ripeness for each batch of fruits rather than being directly correlated with harvest time.

Another measure of oxidation is K_{270} which measures the secondary oxidation products such as carbonyls, aldehydes and trienes. Long storage times and excess heat from refining are known to cause an increase in K_{270} (Elez-Martinez et al., 2005), however, this work aimed to understand how grade, timepoint, and fruit material used in processing could impact secondary oxidation products. Unlike the primary oxidation product measurements, Figures 3.4c and d shows that K_{270} had more clear differences according to grade of fruit (p-value =$2.2x10^{-13}$ for CA and $4.0x10^{-11}$ MX) and processing (p-value =$1.1x10^{-9}$ for CA and $7.7x10^{-7}$ MX) for both regions. However, grade had a greater effect size (f-statistic for CA was 168 for grade compared to 80 for processing and 69 for MX grade compared to 31 for processing), which can be seen visually in Figure 3.4c. The blue shapes (G4) had higher K_{270} values than the shapes in orange; looking within each grade (color) the whole fruit (the "x") is higher than oil made from only the mesocarp (the "circle"). These results could also explain why K_{270} had lower p-values for both regions according to grade compared to PV and K_{232}, secondary oxidation products were formed instead of just primary, which happened to a greater extent in G4 oil compared to G1.

The range of K_{270} for extra virgin oils made from G1 fruits was 0.063-0.220 (Table 3.1). The K_{270} limit for extra virgin olive oils is less than or equal to 0.22 (IOC, 2019; CDFA, 2021; USDA 2010) and less than 0.25 for virgin oils. If the olive oil standards were to be adopted for avocado oil all the G1 in this study would pass as extra virgin. Interestingly, whole fruit oils did have a higher degree of secondary oxidation products than mesocarp (while still within the 0.22 limit). This could be a continuation of what was seen in the K_{232} data, the higher amount of chlorophyll in the whole fruit oil could have helped lead to the formation of secondary products

(further explaining the lack of consistent trend seen in PV and K_{232}). Regardless of the elevated values in whole fruits, all the G1 whole fruit oils fit within the olive oil standard, demonstrating that an extra virgin avocado oil can be made from both whole fruit and only mesocarp. Literature is limited but a similar range of values (0.09-0.21) for cold-pressed avocado oils have been reported (Ramierez-Anya et al. 2018 and Roda et al. 2019).

The ΔK value increases during refining and therefore is a useful parameter for differentiating a virgin/extra virgin from refined or detecting presence of refined oil in unrefined oil. Table 3.1 shows the range of ΔK values; all G1 oils were less than or equal to 0.01, the standard for virgin/extra virgin olive oil (IOC, 2019; USDA, 2010; CDFA, 2021). Our previous study also found that this limit was appropriate and able to differentiate commercially refined oils to unrefined oils (Green and Wang, 2020).

Total phenolics

Polyphenols, or phenols, are antioxidants that can improve stability and extend the shelf-life of oils. These compounds are of particularly high abundance in olive oils, with total phenols content (TPC) at around 150-300 ppm, typically expressed in mg/kg gallic acid equivalents (GAE) or caffeic acid equivalents (Gutierrez et al. 1999, Salvador et al. 2001, and Polari et al. 2018). Forero-Doria et al. 2017 demonstrated that the TPC in avocado oil is much lower than olive oil, which was consistent with our results. Table 3.2 shows that TPC content in our samples ranged from ND-35.16 mg/kg GAE oil, similar to values obtained in Martinez-Padilla et al. 2018. There is a lack of literature on the phenolics in avocado oil extracted solely through mechanical means. When accounting for studies that have also used other extraction methods such as solvent or microwave a huge variety in TPC has been reported, from 1,300 mg/kg GAE

to as low as the non-detectable values seen in this study (Tan et al. 2018, Krumreich et al. 2018, Espinosa-Alfonso et al. 2017).

It is expected that the processing variables of whole fruit and mesocarp in this study would contribute to significant differences in the TPC content in oil. It has been shown that avocado seeds have the highest TPC content, followed by exocarp and then mesocarp (Wang et al. 2010; Tesfay et al. 2010). Our results are consistent with this (Figure 3.5a and b) where the oil samples processed from whole fruit had consistently higher TPC than the oil samples made from mesocarp only. P-values indicated processing had a significant effect on sample differences where the p-value=$1.3x10^{-15}$ for CA and $1.2x10^{-12}$ for MX. Grade also contributed to significant differences between samples but had a lower effect size compared to processing (f-statistic=255 for processing vs 80 for grade for CA samples and f-statistic=87 for MX processing vs 26 for grade. This can be seen visually in Figure 3.5a and b, within each type of oil (whole or mesocarp), G4 had higher phenolics content than G1. While this is a new finding for avocado oil, this is consistent with the published work by Villa-Rodriguez et al. 2011 where they found that the TPC in avocado mesocarp increased significantly with the ripening stage. This was attributed to the relationship between the enzyme phenylalanine ammonia lyase (PAL), which is involved in phenol synthesis, and ethylene production; as ethylene production increases the activity of PAL also increases (Martinez &Whitaker, 1995; Villa-Rodriguez et al. 2011). An important difference between Villa-Rodiguez et al. 2011 and our study is the grade 4 avocados used in our study were at a significantly higher ripening stage. Because the avocados in our study were so overripe, they were degraded and moldy (Figure S3.1), we could also be seeing higher phenols in G4 due to fruit rotting causing large phenols that do not absorb as well at the 725 nm used in this study to decompose into smaller phenolics, which do absorb well at 725 nm. This could also be

responsible for the high standard deviation seen in a couple samples (MX, G1, W late and early season, Table 3.2). Replicates were done by taking unique aliquots of oil and it is possible these aliquots had differing amounts of phenolic breakdown products, contributing to higher standard deviations. More research is needed to chemically characterize the individual phenolics in avocado oil, particularly throughout many ripening stages, to better explain the trends seen here and to determine how ripening impacts phenolics in the seed and exocarp as well as the mesocarp. This may also help explain the large discrepancy seen in TPC content measured in avocado oils studies so far. Phenolics are known for their health benefits and antioxidant capacity, identifying, and quantifying these compounds in high quality unrefined avocado oil could ultimately help the industry bring awareness to their consumer about the health benefits of this product and compare it to other high-value edible oils.

Quality standards for virgin and extra virgin grades

Currently, there are no virgin or extra virgin categories in the proposed CODEX avocado oil standards. However, building on the work by Woolf et al. (2009), quality parameters for the virgin and extra virgin avocado oil grades should be considered either as two separate categories, as is for many of the olive oil standards, or one unrefined category that does not require further refining for human consumption (i.e. crude oil). In either case, it will help differentiate these oils from crude and refined products. The challenge is where the limits should be set given there are many impacting factors such as fruit quality, post-harvest, processing, and storage conditions. Unlike olive oil which has been extensively studied, quality data on avocado oil is much more limited.

In our study, the G1 oils (16 samples total) represent a good quality avocado oil, that should be expected to fit in extra virgin avocado oil standards. For FFA, all 16 of our G1

samples, both whole and mesocarp, fit into Woolf's extra virgin proposed limit of 0.5 % as oleic acid. All fruits used were in optimal G1 condition with no bruising or damage (Figure S3.1), however, in most oil processing situations fruits will not be able to be hand-picked for the best quality furthermore, the best quality avocados are sold as fresh fruits rather than made into oil. By increasing the FFA limit to 0.8 % as oleic acid, which is also reflective the USDA and IOC extra virgin olive oil limit, it could give more leniency in the fruits that can be used to make oil (USDA, 2010; IOC, 2019). For PV, most of our samples were less than 4.0 meq O_2/kg however, there were two G1 samples at around 7 meq O_2/kg. Although all whole fruit and mesocarp oils were still lower than the virgin/extra virgin olive oil limits at 15 and 20 meq O_2/kg (IOC, 2019; USDA, 2010; CDFA, 2021), a PV value of 10 meq O_2/kg might be more achievable for extra virgin avocado oil as this value is expected to increase during storage and shelf-life of the oil.

There are currently no proposed limits for specific extinction values for avocado oil. While USDA and IOC extra virgin olive oil standards for K_{270} and ΔK seem to be fitting for avocado oil, only 10 out of 16 samples met the K_{232} limit of 2.5 for extra virgin olive oil. Thus, K_{232} limit between 2.5 and 3.0 may be more appropriate for avocado oil as this value increases with time and an oil should meet the limit not only at the time of production but throughout its shelf-life. More research is needed to better understand how the K_{232} in avocado oil changes with processing and with time and what oxidation products contribute to the higher values seen from the samples in this study. For all these proposed values, continued research is needed to ensure that oils can meet these parameters and that they are reasonable under proper fruit handling and processing procedure. As the industry continues to grow, avocados for oil productions from different climates and cultivars may not fit in these original limits and standards should be continually updated based on new research data. A significant finding from this study is it is

possible for both whole fruit oils and mesocarp only oils to fit into extra virgin avocado oil standards. This has implications in waste reduction by using the whole fruit instead of only the mesocarp. It also means standard development for avocado oil will be simplified as whole fruit oil and mesocarp do not need separate quality standards.

Conclusions

This study demonstrates how natural variables such as fruit quality grades, growing regions, harvest times, and using whole fruit vs mesocarp in processing impact the chemical composition of avocado oil and oil extractability. It was shown for the first time that oil extraction using whole avocados can meet virgin/extra virgin grade standards. Fruit quality had the biggest effect on FFA, while both fruit quality and processing, to different degrees, impacted oxidation parameters K_{232} and K_{270}. PV was largely unaffected by the variables considered in this study. This data can help with standard development to ensure the established limits for virgin/extra virgin avocado oil are attainable, reasonable and can be achieved. A larger sample size as well as continued research on the combined effects of fruit compositions, cultivars, harvest time, post-harvest and processing on oil yield, quality, phenolics and shelf-life are needed so that processors can make informed decisions on post-harvest and processing procedures to obtain the oil they aim to produce.

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Competing Interests

The authors have no competing interests to declare.

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Tables

Table 3.1. Quality parameters measured in this study (mean ± SD, n=3).

Harvest	*Grade*	*Processing*	*Free fatty acidity (% oleic acid)*	*Peroxide value (meq O_2/kg)*	*K_{232}*	*K_{270}*	*Delta K*
Early Season CA	1	Whole	0.21±0.01	3.33±0.46	2.31±0.22	0.13±0.02	ND
		Mesocarp	0.27±0.04	2.19±0	2.33±0.08	0.07±0	ND
	4	Whole	3.1±0.04	4.19±0.39	2.87±0.04	0.28±0	ND
		Mesocarp	1.16±0.01	5.04±0.11	2.51±0.07	0.19±0.01	ND
Mid Season CA	1	Whole	0.29±0.01	3.24±0.62	2.56±0.03	0.17±0.01	ND
		Mesocarp	0.24±0.01	3.11±0.31	2.17±0.08	0.08±0.01	ND
	4	Whole	4.66±0.03	6.36±1.4	3.07±0.1	0.36±0.01	0.01±0
		Mesocarp	3.72±0.01	3.83±0.44	2.74±0.03	0.24±0.01	ND
Late Season CA	1	Whole	0.14±0.01	2.74±0.69	2.51±0.05	0.16±0.01	ND
		Mesocarp	0.28±0.01	2.78±0.59	2±0.02	0.08±0.01	ND
	4	Whole	2.27±0.02	3.43±0.1	2.69±0.02	0.28±0.02	0.01±0.02
		Mesocarp	1.23±0.01	4.01±0.49	2.13±0.03	0.16±0	ND
Early Season MX	1	Whole	0.17±0.01	7.22±0.71	2.67±0.14	0.22±0.02	ND
		Mesocarp	0.31±0.01	3.18±0.4	2±0.06	0.11±0.01	ND
	4	Whole	3.12±0.01	5.24±0.62	3.39±0.03	0.55±0.02	0.01±0
		Mesocarp	1.92±0.02	4.56±0.36	2.31±0.01	0.32±0.01	0.01±0
Early/mid Season MX	1	Whole	0.16±0.01	3.36±0.53	2.53±0.02	0.17±0	ND
		Mesocarp	0.18±0.01	7.24±0.66	1.63±0.01	0.06±0	ND
	4	Whole	5.86±0.03	8.13±0.73	2.78±0.03	0.54±0.01	0.03±0
		Mesocarp	8.01±0.37	6.76±0.72	2.36±0.03	0.39±0.01	0.02±0
Mid Season MX	1	Whole	0.19±0.01	2.31±0.12	2.41±0.03	0.15±0	ND
		Mesocarp	0.28±0.01	2.17±0.34	1.95±0.03	0.1±0	ND
	4	Whole	0.41±0	2.66±0.23	2.47±0.05	0.16±0.01	ND
		Mesocarp	0.43±0.07	2.77±0.18	1.83±0.02	0.1±0	ND
Mid/Late Season MX	1	Whole	0.15±0.01	2.32±0.11	2.71±0.02	0.18±0.01	ND
		Mesocarp	0.25±0.02	2.18±0.34	2.19±0.03	0.06±0.01	ND
	4	Whole	1.02±0.01	2.63±0.24	2.61±0.04	0.24±0.01	ND
		Mesocarp	1.49±0.01	2.79±0.2	1.83±0.13	0.14±0.01	ND
Late Season MX	1	Whole	0.22±0.01	3.57±0.87	2.65±0.09	0.18±0.01	ND
		Mesocarp	0.28±0	2.76±0.19	2.26±0.04	0.08±0	ND
	4	Whole	1.62±0.02	2.11±0.22	2.71±0.02	0.3±0	0.01±0
		Mesocarp	2.29±0.01	1.91±0.4	2.27±0.03	0.22±0.01	ND

Table 3.2. Total phenol data (mean ± SD, n=3). Samples with trace had concentrations less than 9.8 mg/kg GAE (limit of quantification) but greater than zero.

Harvest	*Grade*	*Processing*	*Total phenols (mg/kg GAE)*
Early Season CA	1	Whole	20.84±3.82
		Mesocarp	trace
	4	Whole	26.8±0.6
		Mesocarp	12.19±1.79
Mid Season CA	1	Whole	21.01±4.21
		Mesocarp	trace
	4	Whole	19.72±1.61
		Mesocarp	11.83±1.79
Late Season CA	1	Whole	11.55±1.33
		Mesocarp	trace
	4	Whole	18.67±1.22
		Mesocarp	11.56±1.65
Early Season MX	1	Whole	20.67±1.54
		Mesocarp	trace
	4	Whole	17.14±9.5
		Mesocarp	8.15±3.58
Early/mid Season MX	1	Whole	17.99±4.37
		Mesocarp	trace
	4	Whole	26±4.47
		Mesocarp	trace
Mid Season MX	1	Whole	17.43±3.81
		Mesocarp	trace
	4	Whole	36.76±5.16
		Mesocarp	14.25±3.18
Mid/Late Season MX	1	Whole	trace
		Mesocarp	trace
	4	Whole	13.16±1
		Mesocarp	10.16±0.35
Late Season MX	1	Whole	19.96±0.93
		Mesocarp	ND
	4	Whole	40.66±10.29
		Mesocarp	11.91±2.91

Figures

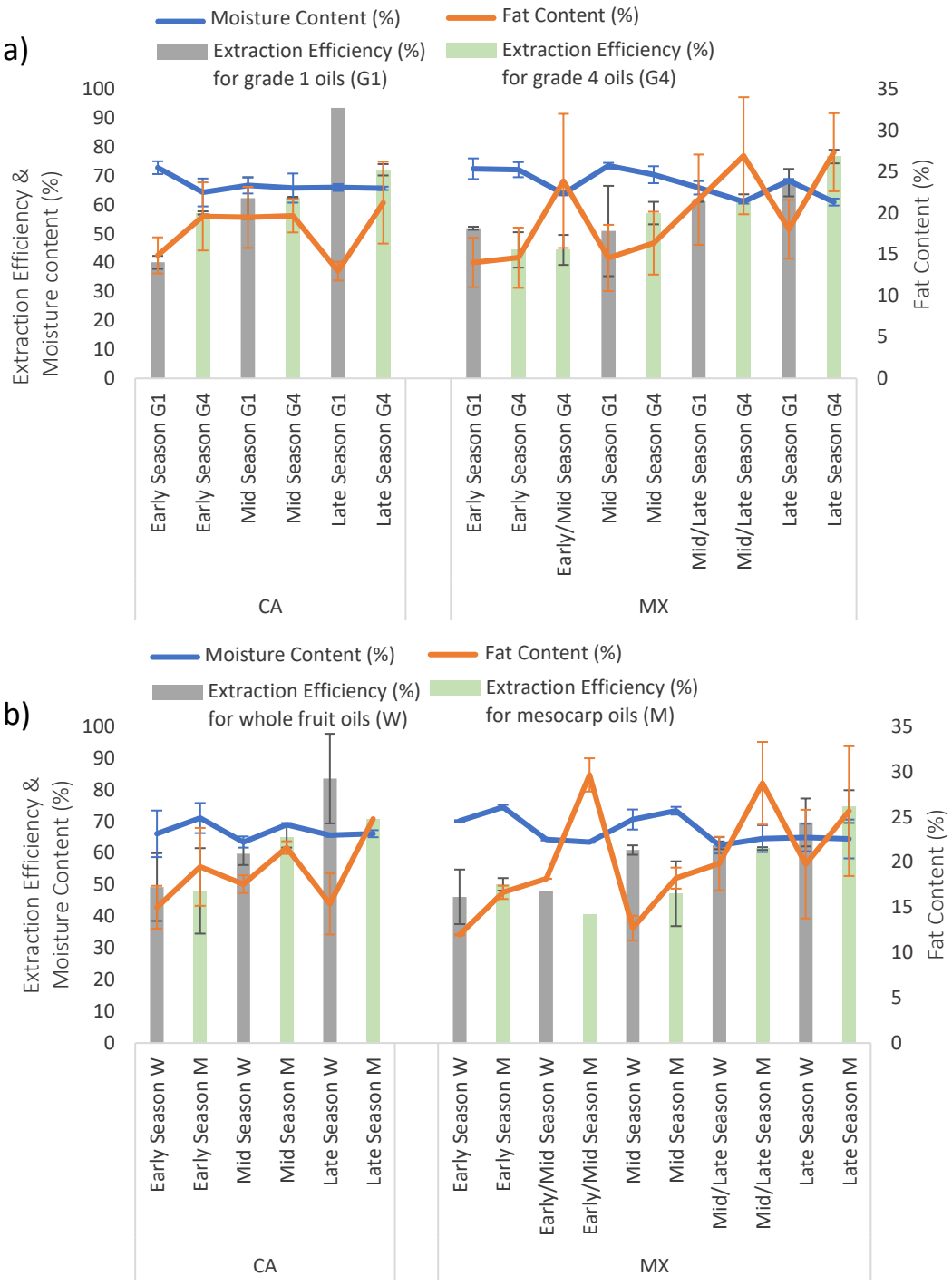


Figure 3.1. a) Plot showing the moisture, fat content, and extraction efficiency separated into location California (CA) and Mexico (MX), timepoint, and grade, where whole and mesocarp samples were averaged across grade of fruit. b) Shows a similar plot but instead sample grades

were averaged across type of fruit used in processing, whole (W) and mesocarp (M). Error bars show standard deviation. Measurements for each sample (defined as an oil with a unique timepoint, location, grade, and either whole/mesocarp). There are no error bars for select lines/bars on MX early/mid season and CA late season due to removing outliers in the data.

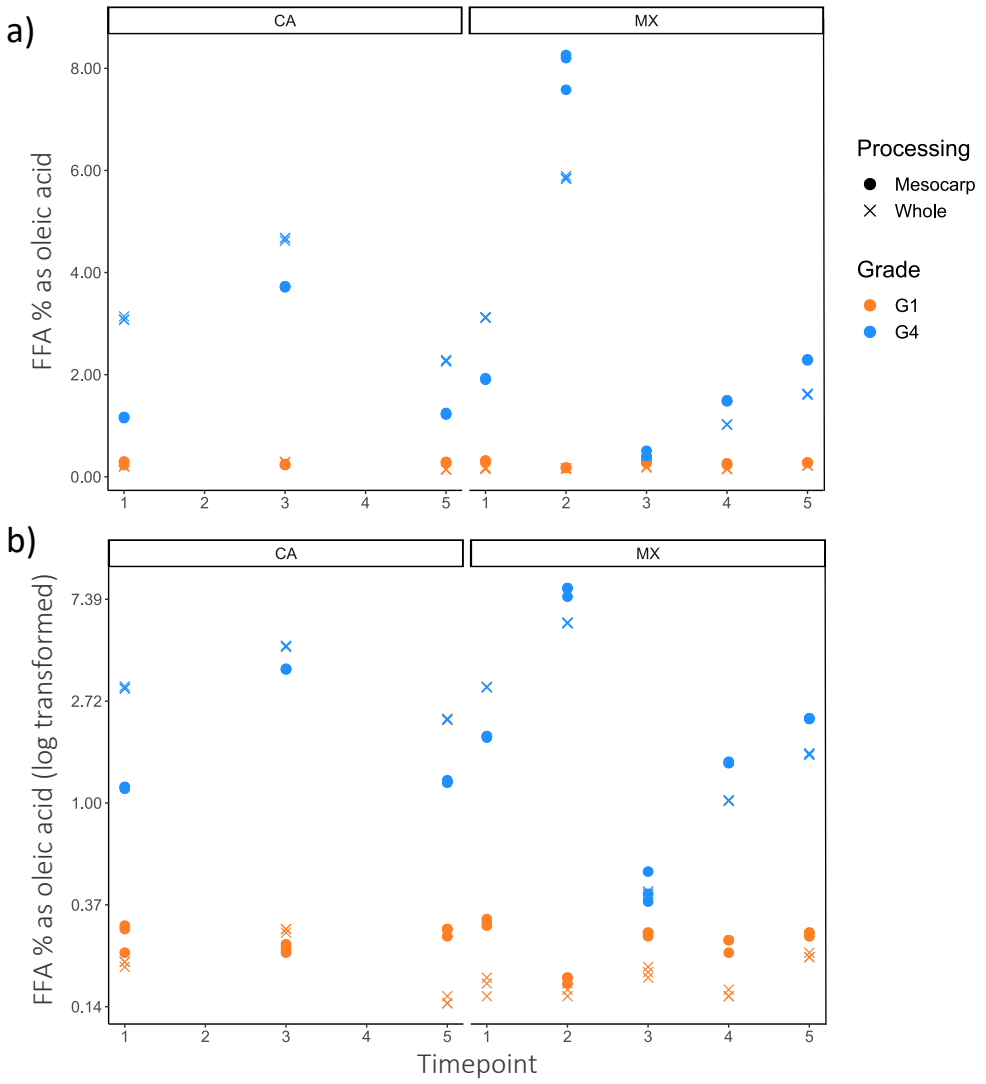

a)
CA
MX
FFA % as oleic acid
8.00
6.00
4.00
2.00
0.00
1
2
3
4
5
Processing
Mesocarp
Whole
Grade
G1
G4
b)
CA
MX
FFA % as oleic acid (log transformed)
7.39
2.72
1.00
0.37
0.14
Timepoint
c)
Whole, grade 1
Whole, grade 4
Mesocarp, grade 1
Mesocarp, grade 4
FFA (% as oleic acid)
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Whole
Mesocarp
1
4
Early Season
Mid Season
Late Season
Early/mid Season
Mid/Late Season
CA
MX

Figure 3.2. a) Two plots showing the free fatty acidity values separated into two plots for each location California and Mexico. Timepoints are numbered according to their place in the season 1, 3, and 5 are early, mid and late season. Mexico has two additional harvest times labeled 2 (early/mid season) and 4 (mid/late season). Samples were run in triplicate; each replicate is plotted to show the variance in the data.. b) Shows the same as FFA data in (a) except the data is log transformed to better show the variance in the data points near zero. (c) A bar graph displays the same FFA dataset, which includes standard deviations (also listed in Table 3.1).

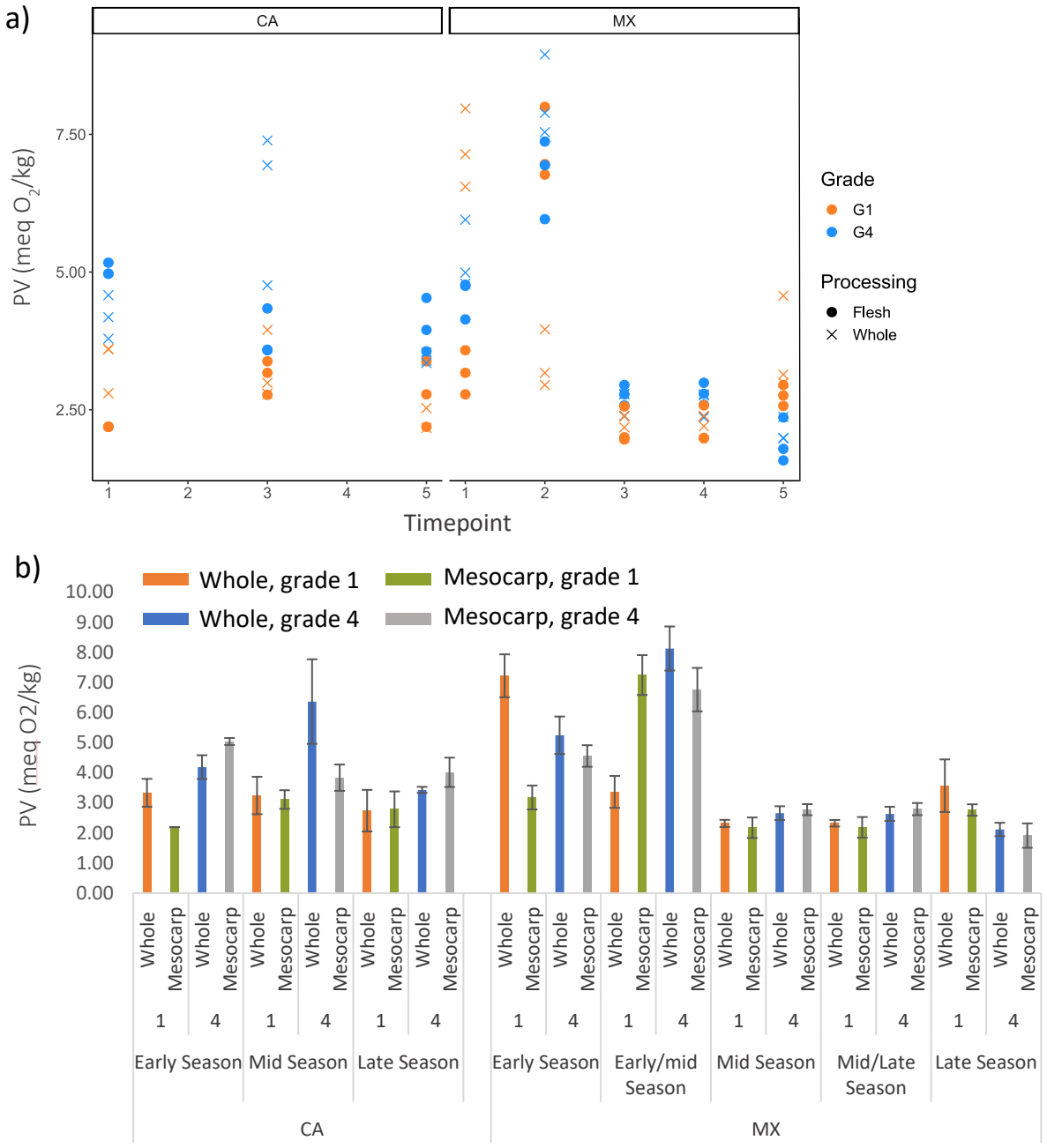
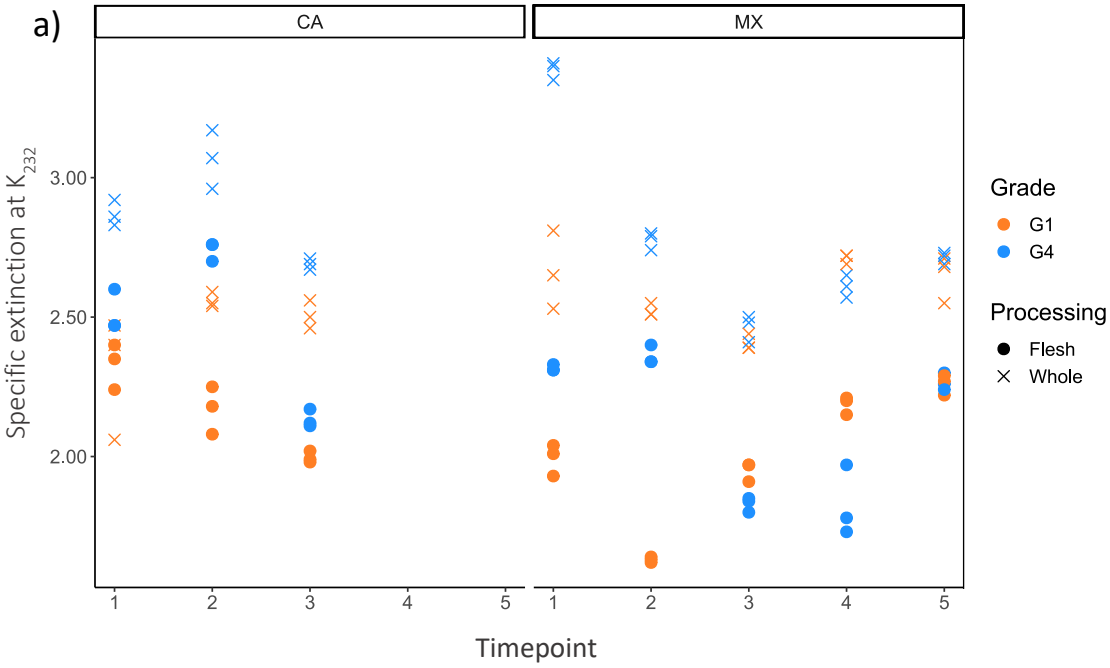
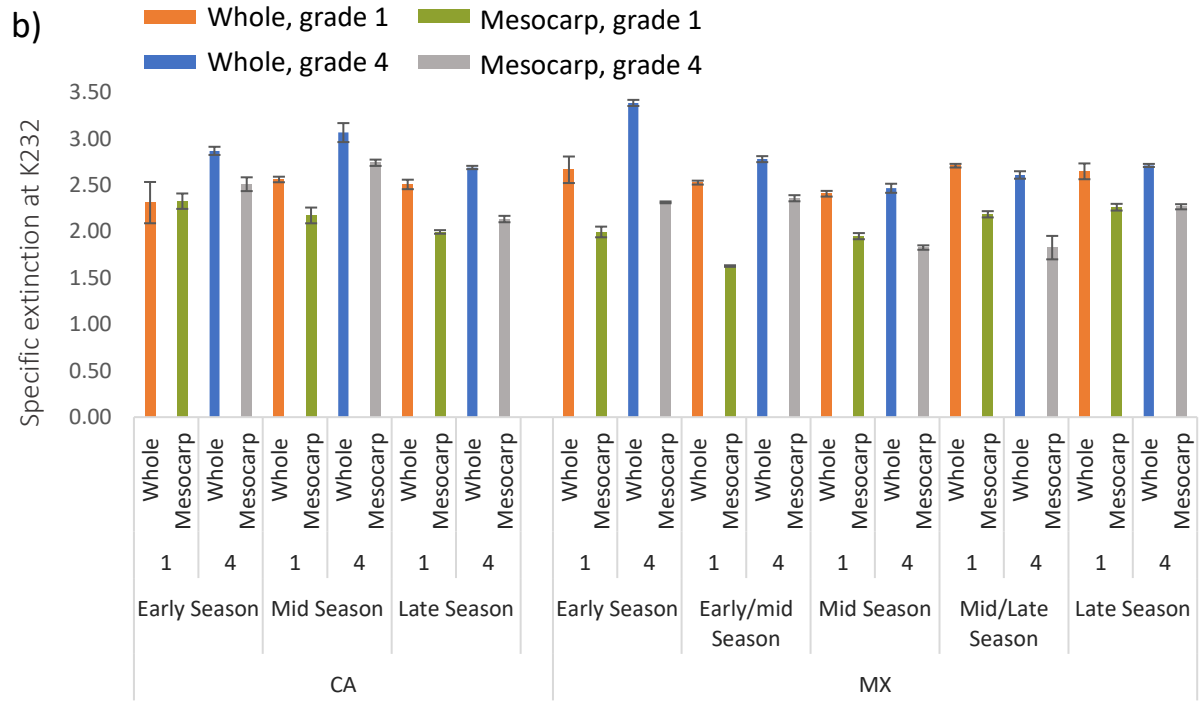


Figure 3.3. a) Peroxide values separated into two plots for each location California and Mexico to visualize trends. Timepoints are numbered according to their place in the season 1, 3, and 5 are early, mid and late season. Mexico has two additional harvest times: 2 (early/mid season) and 4 (mid/late season). Samples were run in triplicate; each replicate is plotted to show the variance

in the data. b) A bar graph displays the same PV dataset, which includes standard deviations (also listed in Table 3.1).





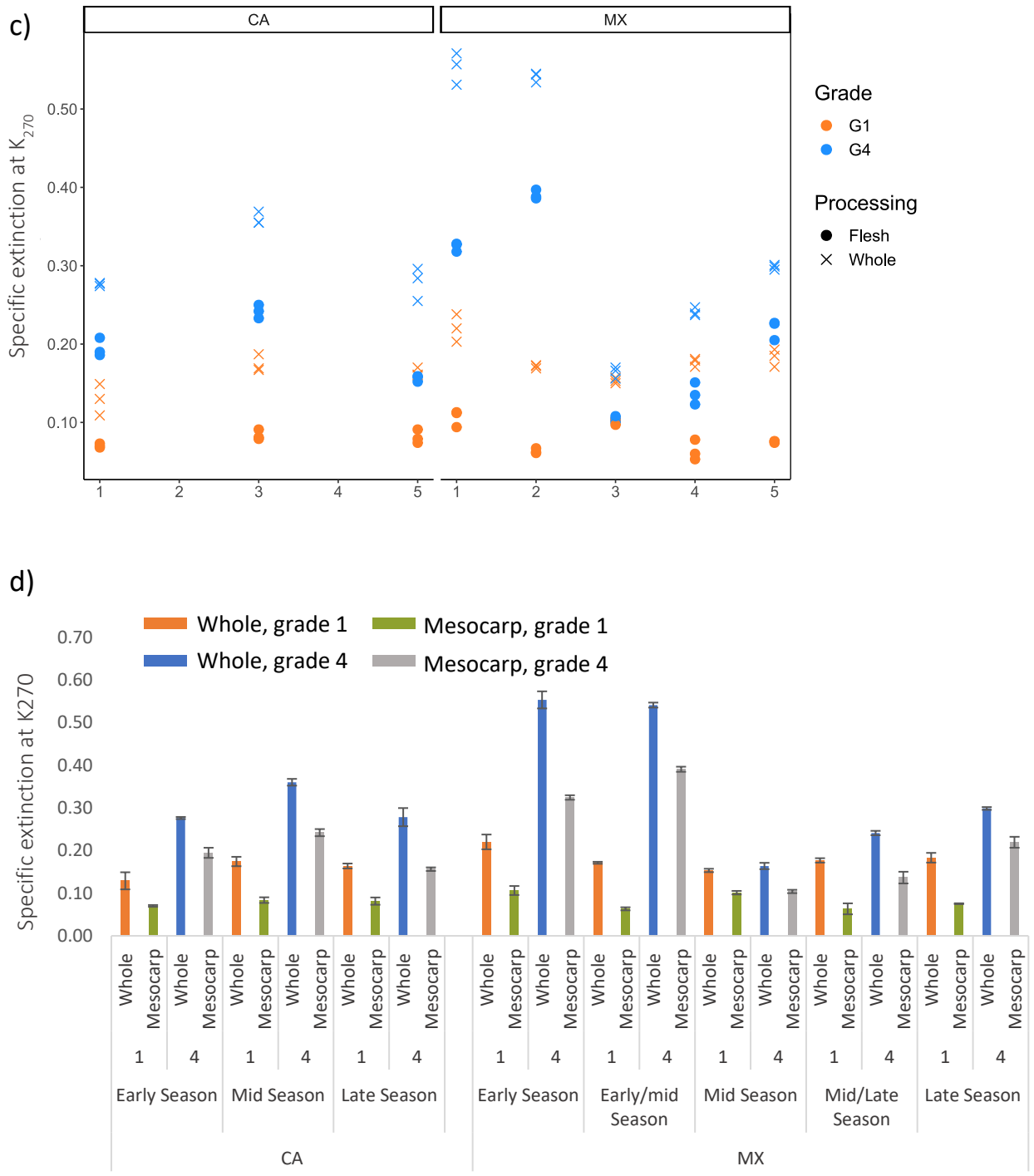


Figure 3.4. a) K_{232}, specific extinction values separated into two plots for each location California and Mexico to visualize trends. Timepoints are numbered according to their place in the season 1, 3, and 5 are early, mid and late season. Mexico has two additional harvest times: 2 (early/mid season) and 4 (mid/late season). Samples were run in triplicate; each replicate is

plotted to show the variance in the data. b) A bar graph displays the same K_{232} dataset, which includes standard deviations (also listed in Table 3.1). c) Displayed in the same was as (a) except values for specific extinciton at K_{270} are shown. d) K_{270} values displayed in the same way as (b).

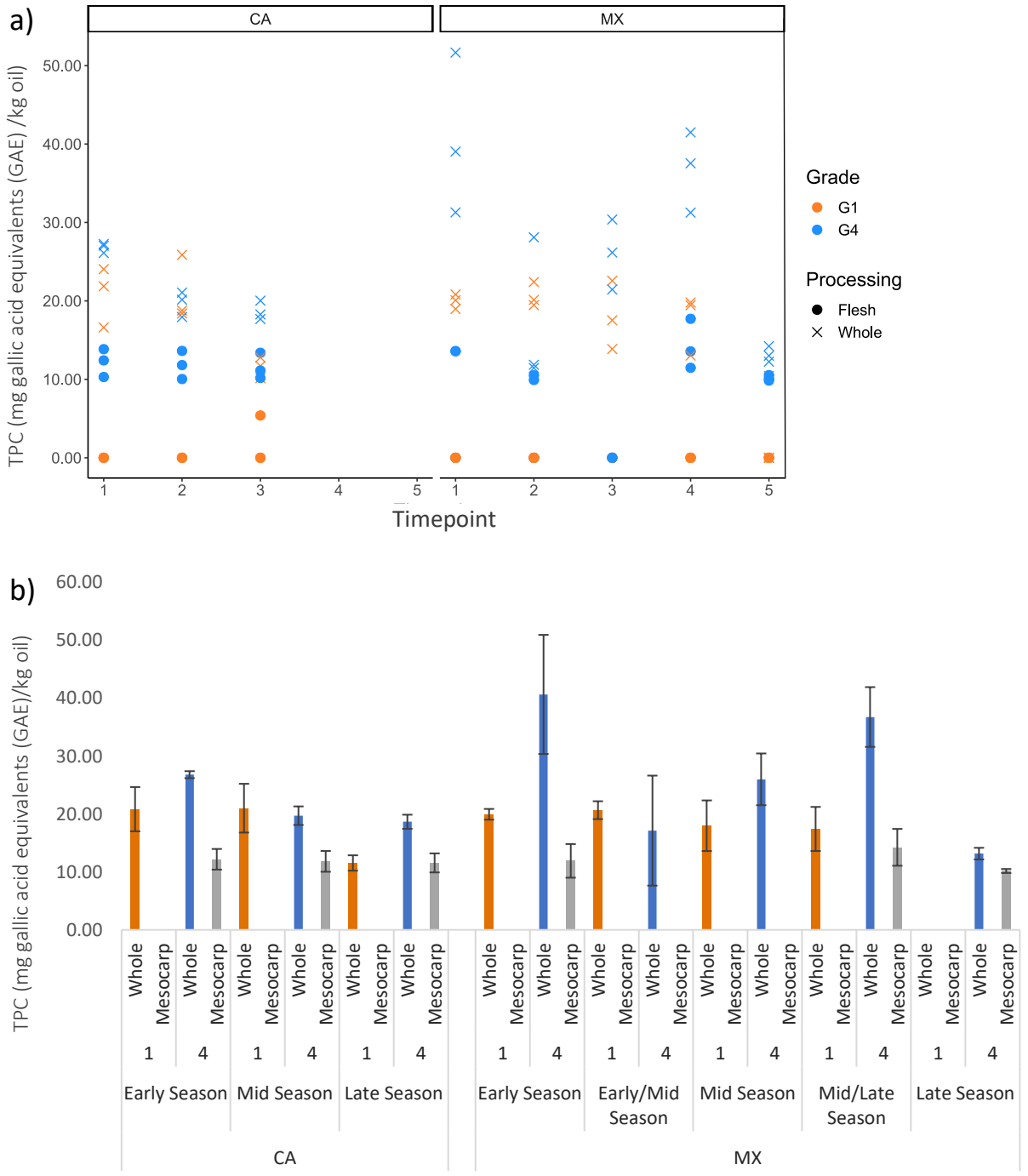


Figure 3.5. a) The total phenol content (TPC) in each oil, separated into two plots for each location California and Mexico. Timepoints are numbered according to their place in the season 1, 3, and 5 are early, mid and late season, respectively. Mexico has two additional harvest times labeled 2 (early/mid season) and 4 (mid/late season). Samples were run in triplicate; each

replicate is plotted to show the variance in the data. b) A bar graph displays the same TPC dataset, which includes standard deviations (also listed in Table 3.2).

Supplementary Figures

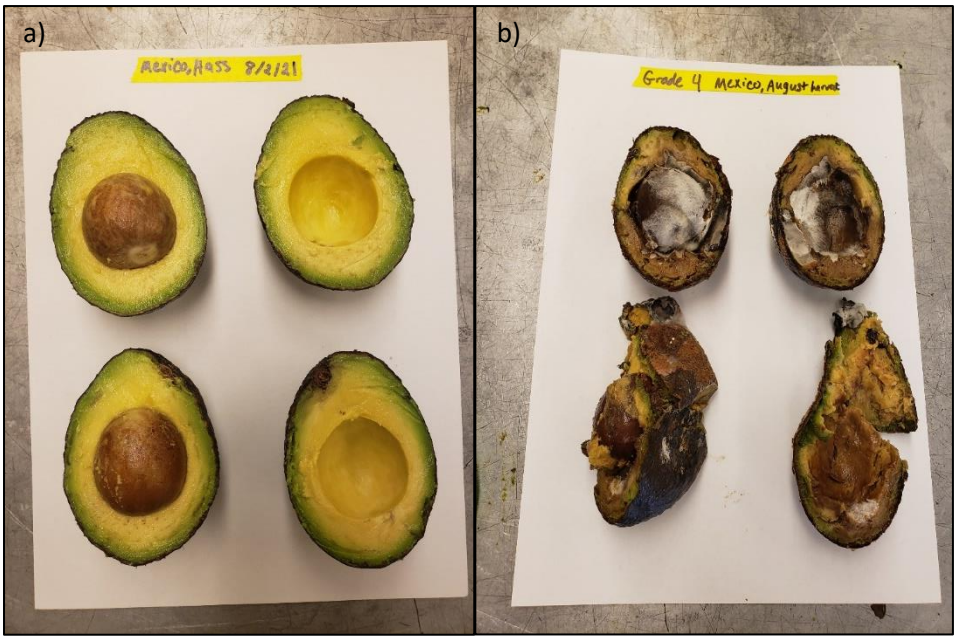


Figure S3.1: Examples of the appearance of grade 1 and grade 4 fruit. Grade 1 (a) is eating ripeness while grade 4 (b) is the worst grade of avocados; these are rotting insect bitten, molding fruits.

Chapter 4

Evaluation of proposed CODEX purity standards for avocado oil

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Abstract

Avocado oil is a high-value product with a growing industry. While facing the ongoing adulteration, standards are being developed with the effort being led by the international organization CODEX Alimentarius. Adequate standards need to accommodate natural variables while also minimizing the likelihood for undetectable adulteration. This study utilizes a comprehensive set of authentic samples for avocado oil and their chemical compositions were compared to proposed CODEX standards. The variables in this study included region, harvest time, cultivar, grade of fruit, and using whole fruit oil versus only mesocarp oil; the chemical parameters analyzed were fatty acids, sterols, and tocopherols. Fatty acids and sterols profiles were most impacted by region and harvest time. And although current standards are heading in the right direction fatty acid and delta-7-stigmastenol and delta-7-avenasterol stood out as parameters that need future research and adjustment. Tocopherols were significantly different for

whole fruit oils compared to mesocarp oils which is an important finding that needs to be considered for avocado oil standard development.

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Background

Edible oils are regulated by standards, or a set of chemical parameters that each oil must fall into to be considered pure or of the appropriate quality. CODEX Alimentarius is an international standard development agency that was formed by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO). Currently, CODEX Committee for Fats and Oils has established an Electronic Working Group (EWG), chaired by Mexico and co-chaired by the USA, and proposed a set of standards for avocado oil (CODEX, 2021b). This is an important step in regulating this product, protecting consumer safety, and ensuring fair global trade and competition in the marketplace. For this set of standards to be useful and fair, there needs to be data and information generated from a comprehensive set of authentic avocado oil samples from all producing regions. Literature studies have focused on fatty acid profile in lieu of sterols, either analyzed for the purpose of exploring extraction methods (Werman and Neeman, 1987; Reddy et al. 2012; Krumreich et al. 2018; Ramirez-Anya et al. 2018; Tan et al. 2018) or only focusing on how a couple specific variables can impact oil such as cultivar and region (Yanty et al. 2011; Ozdemir and Topuz, 2004; Jorge et al. 2015; Manaf et al. 2019; Fernandes et al. 2018; Tan et al. 2017, Slater et al. 1975). These studies are necessary in elucidating how each variable impacts oil, however, in terms of standard development a more comprehensive approach is needed to see the maximum variance within the product. In addition, there is a need to understand how whole fruit oil is impacted by these parameters as the CODEX product definition is “avocado oil may be derived from either the mesocarp of avocado fruit (*Persea americana*) or obtained by processing the whole avocado fruit” (CODEX, 2021b), but there is minimal to no research comparing how whole fruit avocado oil compares to mesocarp only avocado oil.

Fatty acid profile (FAP) is a classic purity parameter used for adulteration detection in oils. It has been more widely studied in avocado oil largely due to the ease of sample preparation. Sterols analysis is a long, arduous analysis, however, they are a valuable parameter that can be used to identify adulteration. Because they are planned to be included in the CODEX avocado standards it is necessary to understand how these compounds change in avocado oil and if any variables should be considered when making standards. Tocopherols are antioxidants naturally occurring in avocado oil and it has been proposed that they could be used to help determine oil purity, however, studies on olive oil show that refining may remove a small portion of tocopherols (Lucci et al. 2020). Gaining insight into oil composition from tocopherols can also be tricky because tocopherols are sometimes added to refined oils as a preservative to increase shelf life, noted on the bottle's label. Even still, CODEX has tocopherol limits for many other vegetable oils including high oleic sunflower and safflower seed oils and soybean oil (CODEX, 2021a).

Several parameters have been shown to impact fatty acid profile, including cultivar (Jorge et al. 2015; Manaf et al. 2019; Fernandes et al. 2018; Yanty et al. 2011), region (Tan et al. 2017), or harvest time (Slater et al. 1975, Ozdemir and Topuz, 2004). Our study aimed to assess how these and several other variables not only impact FAP but also sterols and tocopherols. Since avocado oils can be made from the whole fruit oils or mesocarp, it is necessary to determine if and how this impacts all the above purity parameters so that appropriate limits can be established in the standards. While there are some similarities between olive oil and avocado oil such as high mono-unsaturated fats, unlike olives, avocados fruits are eaten fresh, and this market is prioritized instead of using the fruits for oils. Because of this, avocado oil is made from a variety of fruit grades, ranging from high quality with low defects to low quality with

significant damage and rots. It is also important to study if and how these drastic changes in fruit quality impact the purity parameters, so the established standards are realistic and practical to be adapted by all producers around the world.

This project used two different experimental designs to assess post-harvest variables that could impact purity parameters. Experimental design one, previously described in Green and Wang (2022) included the variables harvest time, country of origin (California, USA or Michoacán, Mexico), grade of fruit, and processing using whole fruit of mesocarp. Experimental design two accounted for harvest time, region of origin (two different locations in California), fruit cultivar, and processing using whole fruit or only mesocarp. This is the first study that has looked at how using whole fruit or mesocarp oil impacts these chemical parameters and to the best of our knowledge, one of the most comprehensive and systematic studies on authentic avocado oil. By analyzing samples from a wide range of harvest times, with multiple growing regions, as well as varying the types of fruit used, it can give information on the expected maximum variation seen in avocado oil chemical composition, which allows for the current proposed CODEX standards to be evaluated and modifications to be considered.

Materials and Methods

Experimental design

This study has two primary experimental designs. The first was previously described in Green and Wang (2022) and is shown in Figure 4.1a. In brief, Hass avocados were collected from California, USA (CA) and Michoacán state in Mexico (MX) throughout the harvest season 2021. Fruits were collected from three different grower lots for each timepoint CA (three timepoints) and MX (five timepoints). Two grades of fruit were processed into oil from each region/harvest time: Grade 1 (G1), high-quality fruits, and grade 4 (G4), low-quality fruits. Fruits

were ripened at 20°C and 80-90% humidity with apples as an additional ethylene source. Grade 1 fruits were ripened to eating firmness while grade 4 fruits until severe rots and decay occurred, Figure 4.1a.

The second experimental design focused on fruit from California and the variable cultivar was used in place of fruit grade, shown in Figure 4.1b. Researchers at the University of California, Irvine harvested fruit from their avocado trees throughout the season of 2020-2021 from two CA locations, Lindcove and Irvine. Lindcove had a shorter season with three fruit collections, while Irvine had six. Two cultivars of fruit, Hass and Carmen, were collected at each time point. Three biological replicates (trees) were taken for each cultivar when available; in some instances, only two trees were available for harvesting. Once ripened in the same way as the G1 fruits from design one, above, each cultivar was processed into oil using either the whole fruit or only the mesocarp/flesh

Avocado oil extraction

Extraction process for design one was previously described in Green and Wang (2022) and a schematic is shown in Figure 4.1a. In brief, a laboratory Abencore oil mill (MC2 Ingenieria y Sistemas S.L., Seville, Spain), was used to extract oil from either the whole avocado fruit or only the mesocarp. First, a Schneider Electric (GV2 MC01) crusher processed the avocado whole fruit or mesocarp samples into paste, then 700 g of each paste was malaxed for hour at 50°C with 10% w/w talc and 3 mL of Novozyme Pectinex Ultra Olio enzyme. Once malaxed for 30 min, 140 mL of water was added. The entire mixture was then centrifuged for 3-4 min, oil was decanted and then re-centrifuged using a benchtop fixed-rotor centrifuge at 10,000 rpm for 10min. Oil was stored in a dark freezer at -20°C until chemical analysis.

Fruit from design two was extracted using hexane solvent extraction instead of extraction mill. Avocados (either whole fruit or mesocarp) were crushed using the Abencore-mill crusher and then spread on aluminum foil and dried an oven at 105°C for 12 h or until all water had evaporated. Dried samples were ground using a food processor and the sample material was extracted using the Buchi Universal Extractor E-800 with *n*-hexane. Once the extraction was complete any remaining residual solvent was evaporated, and oils were stored in a dark freezer at -20°C until chemical analysis.

Fatty acids profile

The IOC official method for the determination of the fatty acid methyl esters by gas chromatography (COI/T.20/ Doc. No 30/Rev.1, 2017) was used for fatty acid profile analysis with some modifications. Approximately 20 µL of oil was mixed with 3 mL heptane. Then 200 µL of methanolic KOH (2M) was added and mixed well for 1min. Samples were left to sit at room temperature until the top solution was clear, which was then filtered with a 0.45 µm PTFE filter for analysis. The GC-FID analysis was conducted on an Agilent 7890A GC (Agilent Technologies) using a 90 m × 250 µm × 0.25 µm DB-FastFAME capillary column (Agilent Technologies) to achieve the separation of individual fatty acids. Helium was used as a carrier gas at a flow rate of 1.9 mL min^{-1} with an injection volume of 1.0 µL and a split ratio of 30. The injector temperature was held at 260 °C. The GC oven program was initially held at 75°C for 1 min; then ramped at 35°C min^{-1} to 200 °C and held for 14 min, followed by a ramp of at 2.5°C min^{-1} to 210 °C, which was held for 5 min. The last ramp was at 12°C min^{-1} to 230°C and held for 20 min, giving a total run time of 49.2 min. The FID temperature was 260°C. The detector gas consisted of hydrogen (flow rate: 40 mL min^{-1}), air (flow rate: 400 mL min^{-1}), and helium make up gas (flow rate: 25 mL min^{-1}). Peak identification was performed using a 37-component

FAME reference standard mix (MilliporeSigma).

Sterols profile

Sterols content was analyzed using a combination of both Mathison and Holstege (2013) and the Phenomenex determination of sterols in olive oil (TN-0114) with modifications. The unsaponifiable fraction was prepared by drying 20 uL of internal standard 0.2% α-cholestanol ethyl acetate solution before adding 200 mg of avocado oil sample and 1.5 mL of 2M KOH in 95 % ethanol. The mixture was capped and heated in a heating block at 80°C for 25 min, mixed and heated for a second 25 min. Samples were removed from heat block and 13.5 mL DI water was added and gently mixed before loading onto a Phenomenex Strata DE SLE cartridge, 60 cc tube, followed by two 1 mL rinses. After waiting 15 min, the extract was eluted with five, 15 mL portions of diethyl ether. Eluent was passed through a syringe packed with glass wool and 6-7 g sodium sulfate. Eluents were dried using a rotary evaporator and then placed in an oven at 100C for 10 min to ensure all water was completely removed before moving on to the SPE step. Dried extracts were reconstituted with 5 mL hexane. Next, the silica SPE columns (6 mL, 1 g sorbent, Agilent brand) were conditioned using two, 6 mL hexane rinses followed by 1 mL of 0.2M KOH in 98% ethanol, followed by an additional 5 mL hexane rinse. Each sample was then loaded onto the SPE column and 85 mL of hexane: diethyl ether (98:2) was used to wash the column at 2 mL/min. The sterols fraction was eluted using 5 mL of hexane: diethyl ether (80:20) followed by 5 mL of hexane: diethyl ether (60:40). Extracts were dried in a rotary evaporator and if needed placed in an oven at 100°C for 5-10 min to evaporate any remaining water. Finally, 250 μL of the silylation reagent (pyridine/hexamethyl disilazane/trimethylchlorosilane, 9:3:1, v/v/v) was added to prepare the trimethylsilyl ethers for GC injection. The GC-FID analysis was conducted on an Agilent 7890A GC (Agilent Technologies). A 30 m × 0.25 mm × 0.25 μm DB-5 capillary

column (Agilent Technologies) was used with an injection volume of 1.0 µL and helium as the carrier gas at a flow rate of 1.2 mL min^{-1}. The injector temperature was held at 280 °C at a split ratio of 5. The GC oven program was held isothermally at 150 °C for 8 min; then ramped at 20 °C min^{-1} to 290 °C and held for 20 min to obtain a total run time of 37.33 min. The FID temperature was 300 °C. The detector gas consisted of hydrogen (flow rate: 30 mL min^{-1}), air (flow rate: 400 mL min^{-1}), and helium make up gas (flow rate: 25 mL min^{-1}). Peak identification was carried out with standards campesterol, stigmasterol, β-sitosterol (MilliporeSigma) and by comparing the generated chromatograms against the sample chromatograms provided in the IOC official method and their relative retention times while the quantification was performed using the peak area and concentration of the internal standard.

Tocopherol analysis

Tocopherols were determined according to Gimeno et. al. (2000) with some modifications (Green et al., 2020). Oil (40 µL) and hexane (160 µL) and were briefly vortexed. The internal standard, α-tocopherol acetate in ethanol at a concentration of 300 µg/mL, was added in addition to 600 µL of methanol. The sample was vortexed for one min and centrifuged (5000 rpm, 5 min, Beckman GS-15R). Samples were stored at -20°C for two hours to allow oil to separate from the organic phase. The organic extract was filtered (0.45 µm, nylon). Analysis was performed on an Agilent 1290 Infinity II LC system with a diode-array detector using an Agilent ZORBAX Eclipse Plus C18 column (3.5 µm, 3 x 100 mm). The mobile phase was methanol:water (96:4), isocratic. A 20 µL injection volume and flow rate of 1.0 mL/min were used giving a total run time was 12 min. DAD signal was recorded at 292 nm. Standards α-tocopherol, δ-tocopherol, γ-tocopherol, and α-tocopherol acetate were purchased from MilliporeSigma. HPLC grade methanol, HPLC grade hexane, and reagent grade ethanol were

purchased from Fisher Scientific.

Statistical analysis

Data was visualized using hierarchal cluster analysis and principal component analysis using OriginPro2016. Statistical analysis was performed using ANOVA in R, with significance being determined by the F-statistic at an alpha level of 0.01. This was used instead of 0.05 to minimize Type 1 error from using multiple ANOVAs.

Results and Discussion

Fatty acid profile

Comparison of fatty acids to proposed CODEX standards

The ranges of fatty acids profile the seen across all samples, 68 in total after averaging across biological replicates, in this study are listed in Table 4.1 and compared to the current proposed CODEX standards (CODEX, 2021b). Table 4.1 illuminates a primary finding; the majority of fatty acids in this study have values that span the ranges in the proposed standards. For example, the samples had an oleic fatty acid (C18:1) amounts between 49.74-71.41%, where the CODEX proposed standard range is 42.0-75.0%. The fact that such a wide variety of oleic acid content was seen in the samples indicates that the variables in this study adequately accounted for much of the natural variance that occurs in avocado oil. It also indicates that the standards for oleic fatty acid likely reflect ranges seen due to natural variation.

However, there were multiple cases where the values seen in this study did not fit into the current proposed standards. For C16:0 there were 1.5% of total samples and for C16:1, 2.9 % of total samples, that were outside their respective 11.0 % and 4.0 % limits. In the case of palmitic fatty acid (C16:0) 1.5% was only one sample was outside the range, thus it was likely an anomaly. For palmitoleic (C16:1), the couple samples below the 4.0 % CODEX limit could also

likely be considered outliers. However, the maximum value seen in our samples was 10.03 % for palmitoleic, compared to the CODEX max of 17.1 %. From an adulteration detection perspective, most vegetable oils have low C16:1 and lowering the 4.0 % limit further would risk avocado oil adulterations getting by, but there is little risk with having a maximum value of 17.1 %. All the samples had linoleic acid (C18:2) below the proposed limit of 19.0 % with an exception of late harvest Irvine samples from experimental design two (5.9 % of samples). Because there is a wide range of linoleic fatty acid in vegetable oils and potential adulterants (CODEX, 2021a) keeping this standard with as narrow limits as possible will help minimize undetected adulterations. C17:0 and C17:1 are not always quantified or reported, however, 5.9% of samples had C17:1 values at 0.11 % rather than the limit of 0.1%. It would be beneficial to raise this limit slightly, so samples do not fail due to this fatty acid.

A primary question from our 2020 study (Green and Wang, 2020) was what causes variation in stearic fatty acid in avocado oil. It was hypothesized that some of the variables accounted for in this study might be able to provide an explanation for higher values seen in that study. Although the C18:0 ranges seen in this study are within standards, we only saw a maximum of 0.68 % compared to the 1.3 % maximum in current proposed standards. We have found that stearic fatty acid is often elevated (around 1.0-2.0 %) in commercial samples compared to the authentic samples analyzed in this study (unpublished data). Because other common avocado oil adulterants have higher stearic fatty acid values (CODEX, 2021a) it is important that an appropriate limit is developed so adulteration does not go undetected. A future study testing oils before and after refining could provide insight, however, more research is still needed to understand if this is a phenomenon due to natural variance or if it is simply due to adulteration of many commercial samples. If the results from this study were to be used to

develop an upper limit for stearic fatty acid, it would be 0.7-0.8 % maximum, which is consistent with literature (Ozdemir et al. 2004, Tan et al. 2017, Berastagi et al. 2012, Maldwala et al. 2012).

The fatty acid profile is the most common method used to assess oil purity, resulting in more literature (the following list is not comprehensive) on FAP than other purity parameters (Werman and Neeman, 1987; Reddy et al. 2012; Krumreich et al. 2018; Ramirez-Anya et al. 2018; Tan et al. 2018; Yanty et al. 2011; Ozdemir and Topuz, 2004; Jorge et al. 2015; Manaf et al. 2019; Fernandes et al. 2018; Tan et al. 2017; Slater et al. 1975; Berastagi et al. 2012; Bora et al. 2001; Maldwala et al. 2012). However, if current literature were to be used to determine standard ranges, it would be too wide to allow avocado oil to be authenticated. This is partially due to the analysis of specific regions and cultivars that are not widely used for oil production or have unique climatic conditions. Manaf et al. (2019) determined the FAP of avocado oil from five cultivars in Indonesia, seeing ranges from 21.69-55.64 % for oleic fatty acid, a stark contrast from the proposed standards in Table 4.1 and from the samples in this study. This highlights why it is necessary to assess the importance of some of these natural variables and to analyze oils from new regions, cultivars, etc. to determine if and how standards can accommodate them, which is discussed further, below.

Variables that impact fatty acids

To gain insights on which variables have the biggest impacts on avocado oil fatty acids, Figures 4.2 and 4.3 visualize the data demonstrating what variables are the most important to consider when developing and harmonizing standards. For clarity, each experimental design was visualized separately since they each accounted for different variables.

Figure 4.2a shows the hierarchal cluster analysis, where it is evident that samples clustered based on region (with MX dominating the first five clusters) and CA dominating the

sixth cluster, in red. Within Mexico there is clear separation based on harvest time. Whole fruit versus mesocarp and grade 1 vs grade 4 fruits are not driving separation. This is an interesting finding as it has been shown that the fatty acid profile differs between the parts of the avocado (Bora et al. 2001). However, because there is so little oil in the exocarp and seed, whole fruit oil and mesocarp only oil do not have major differences in their fatty acid profile. This is relevant to standards as the definition of avocado oil has been debated and is currently defined by CODEX as oil that is derived from either the whole fruit or the mesocarp. Our results indicate that oil extracted from whole fruit, mesocarp, or a combination of these two can share the same FAP standards.

Because region and harvest time contributed to the biggest differences amongst the samples FAP, Figure 4.2b further looks at the separation of these two variables. The MX samples are largely located below the x-axis, with early and early/mid MX samples having higher amounts of C16:0, C16:1, and C18:0. Mid-season MX had the strongest correlation with C18:1, which is a trend consistent with what was shown in Slater et al. 1975 where the oleic fatty acid content increased throughout the season and then decreased again in late season. Both Slater et al. (1975) and Ozdemir et al. (2004) also saw that as oleic acid increased palmitoleic decreased and vice versa. The late season MX samples are clustered with most of the CA samples, which are above 0 on the x-axis and correlated with C18:2 and C18:3, along with several other lower concentration fatty acids.

Figure 4.3 summarizes the results from design two, which zooms in on two locations in California with different climates. Irvine, which is along the coast with moderate temperatures throughout the year and Lindcove, which is inland at a higher elevation with large temperature fluctuations throughout the year. Two different cultivars were compared, Hass (fruits in design

one) and Carmen, officially known as Hass-Carmen. As the name suggests this cultivar is related to Hass but flowers at different times. Since this cultivar could be used for oil and has the potential to provide a larger crop in the off-season it was also investigated.

Similar trends can be seen in this design where differences in samples are dominated by harvest time and region with whole fruit/mesocarp having little impact on FAP, Figure 4.3a. Interestingly, season and region also seem to be overcoming the effects of cultivar. In this case it is likely because the two cultivars are genetically related as previous studies like Fernandes et al. (2018) and Manaf et al. (2019) have shown that cultivar can significantly affect FAP. Figure 4.3b shows most of the Lindcove samples are located below the 0 on the X-axis and clustering can be seen when accounting for both timepoint and region, where late season Irvine samples are correlated with C18:2. Interestingly, many early season samples (very early Irvine and early Lindcove) are correlated with C16:0, C16:1, and C18:0 just like in design one. There were significant differences in C16:0 and C18:0 based on region for design one and two, with the addition of C16:1 for design two. Significant differences in C16:0 and C16:1 from harvest time for design two were also found, highlighting how region and harvest time are intertwined. Previous literature has shown temperature is a major factor that impacts fatty acid synthesis, which is related to both region and harvest time (Ferreya et al. 2016 and Kaiser and Wolstenholme 1994). It will be important in the future that new producing regions have oil evaluated throughout the harvest seasons to determine how significantly their fatty acid profile is affected and if the current standards accommodate those oils.

Sterols profile

Comparison of sterols to proposed CODEX standards

Sterols analysis is more difficult and time consuming than fatty acid profile and therefore there is minimal literature on avocado oil sterols. However, because sterols are included in the CODEX standards, thorough analysis of the variables that can impact them using an adequate set of authentic samples is needed (CODEX, 2021b). This is the first study to do a comprehensive analysis of sterols in avocado oil accounting for this breadth of natural variables. Overall ranges seen in this study for sterols are listed in Table 4.2. No cholesterol or brassicasterol were detected in any samples.

Stigmasterol was not detected in many samples even though the minimum limit is currently 0.3 %. It should be considered that this minimum be lowered to not detected (ND) to accommodate samples, which 33.8 % did not meet current ranges. Considering most adulterant oils have higher stigmasterol, this adjustment is feasible. For campesterol and delta-5-avenasterol, there were samples with values above the CODEX maxima. There were 11.8 % of samples that had campesterol values greater than 8.3 % primarily including early/very early season Lindcove and Irvine. Due to the number of samples with higher values, this data initially suggests that the range should be reconsidered, however, the risk with doing this is other potential adulterant oils have higher amounts of campesterol. ANOVA confirmed that campesterol was significantly impacted by both harvest time and region. It is possible that the effect of timepoint poses minimum risk as not many fruits are harvested in the early/very early season as was done in this study. However, if this standard range does not change, authentic oil made from early harvest fruits could have campesterol values above 8.3 % and therefore fail to meet the purity requirement of avocado oil. There were also several California samples with values above 8.0 % for delta-5-avenasterol. Most potential adulterants have delta-5-avenasterol values with similar to or less than avocado oil, thus, increasing the limit to 8.5 % to

accommodate more CA samples would have minimal impact on the ability of this standard to detect adulteration.

Both clerosterol and beta-sitosterol currently have a couple different proposed minimum values that have not been agreed upon. Clerosterol is not quantified in other vegetable oils, however, due to its higher concentration in avocado oil it has been proposed to be included in the avocado oil standards. All samples were above 1.47 %, making the 1.0 % proposed minimum more reasonable than the 0.6 % minimum. However, there should be more discussion on the upper limit of this standard. There were 39.7% of samples that had clerosterol values above 2.0 % (only one was above 3.0% and is an outlier). A clerosterol limit that goes up to 3.0% would be more accommodating, particularly for the CA samples from experimental design two which had overall higher values compared to design one, listed in Table S4.5 and S4.6 (design one) and Table S4.7 and S4.8 (design two). In the case of beta-sitosterol this study saw a narrow range compared to the proposed limits. All samples were above 80 %, which is significantly higher than the 71 % proposed minimum and more in alignment with the 79 % proposed minimum. This is particularly important because avocado oil is characterized by having a high amount of this sterol. And because most adulterant oils have lower values of beta sitosterol (CODEX, 2021a). A low minimum value would allow for more undetected adulterations.

Narrow ranges were also seen for both delta-7-avenaterol and delta-7-stigmastenol in this study compared to the proposed values. And, like beta-sitosterol it is particularly important that the standard range for these two sterols is as narrow as possible because most adulterant oils have significantly greater amounts of both delta-7 sterols. We only saw a maximum of 0.09 % for delta-7-stigmastenol and 0.27 % for delta-7-stigmastenol compared to their proposed maxima of 1.0 or 3.5 % and 1.5 %, respectively. It is also worth noting that most of the samples in this

study had nondetectable levels of both of these sterols. A similar trend has been seen with the delta-7 sterols as with stearic fatty acid. There has been reports of commercial samples (most of which are refined) having higher of both delta-7 sterols compared to the laboratory-made samples. Refining could play a role, which is not accounted for in this study. However, this study does account for both cold-pressed and solvent extracted oils. More investigation is necessary to determine the degree of discrepancy from processing influence or adulteration in the commercial samples tested. If the samples in this study are being used as a guide an upper limit of 1.0 % or under seems most appropriate for both delta-7-stigmastenol and delta-7-avenasterol.

Variables that impact sterols

The sterols results are visualized in Figures 4.4 and 4.5 for experimental designs one and two, respectively. Unlike the fatty acid profile, there was less variance seen in the sterols profile across samples, which is also shown by the y-axis only going up to 3.0 in Figure 4.4a. There were no clear clusters that could be attributed to a specific variable. The group in pink/orange on the far right contains only early season samples from both CA and MX; the other main distinct group on the far left in red/green only contains grade 1 samples. There are also some mesocarp samples located together within the largest dark blue cluster. Because all these variables seem to play a role to some degree in sample differences, two different PCA plots were made. Figure 4.4b shows a plot labeling samples according to region and timepoint, while Figure 4.4c according to grade and processing. These plots together demonstrated timepoint and region play a bigger role in sample clustering compared to grade and processing, Figure 4.4b showing mid-season CA, mid/late MX, and Late MX are correlated with beta-sitosterol.

Similar trends are seen in experimental design two. The variance across samples was also narrow (Figure 4.5a) and the dendrogram shows clustering primarily by harvest time, early and

very early samples on far left, and by region, with Irvine largely in the four clusters on the right. Minimal to no clustering is seen based on the two cultivars or processing material. Figure 4.5b provides more detailed information on how each sterol correlates to samples. Early, early/mid, and mid-season Irvine samples were correlated with delta-5-avenasterol, while late and mid/late season Irvine samples with beta-sitosterol (similar trends to Figure 4.4b). Analysis via ANOVA revealed that unlike other sterols, there was a significant difference in stigmasterol content in the whole fruit (higher) compared to the mesocarp. This was consistent across designs one and two however, interestingly, the range of values seen for stigmasterol was quite different; design one saw a range of ND-0.5% while design two was from 0.5-1.4%. Overall, like fatty acids, the variables that contribute to the most significant differences in sterols are the time and location of the harvested fruit. This leads to the same recommendation as the fatty acids but with greater needs due to lack of data: it will be important in the future that new producing regions or fruits harvested at atypical times are analyzed for the sterols profile to ensure standard compliance.

Tocopherols: implications for including in purity standards

Tocopherols are naturally occurring antioxidants found in avocado oil, the primary compound being alpha tocopherol or vitamin E. However, they have not been well studied in avocado oil. Tocopherol content was only measured for the design-one samples (Table 4.3, 32 samples in total), because they were extracted via pressing rather than with heat and solvent, which has the potential to affect tocopherol concentration to varying degrees (Lucci et al. 2020). The most recent draft for CODEX avocado oil standards (CODEX, 2021a) has included proposed values for tocopherols and opened discussion on the inclusion of these compounds in standards. Proposed values include 50-450 mg/kg for alpha tocopherol (same range for total tocopherol content), 10-20 mg/kg for gamma and ND-10 mg/kg for delta (CODEX, 2021a). Our

results show that all samples fit in the range for alpha tocopherol, although our max was under 200 mg/kg. This is comparable to literature where the maximum reported value that we have found is 202 mg/kg of alpha tocopherol (Corzinni et al. 2017). The proposed maximum of 450 mg/kg is significantly larger than what has been reported so far, unless this is to also accommodate samples that have tocopherols added for shelf life. Gamma/beta were not detected in 75% of mesocarp samples, which although the 20mg/kg max is appropriate for those oils, whole fruit oils had values as high as 30 mg/kg. Delta tocopherol was only detected in whole fruit oils and in some cases had a higher concentration compared to gamma/beta tocopherols. Fernandes et al. (2018) also saw high levels of delta tocopherol, 26.84 mg/kg, where Hass fruits were pressed using the same Abencore system as used in this study.

To see trends in the variables affecting the samples, data was visualized in Figure 4.6, which shows strong, near perfect clustering of samples according to processing oil using whole fruit or mesocarp. The whole fruit oils are further clustered into grade 4 and grade 1. In Table 4.3, we can see whole fruit oil contains more gamma/beta (quantified together, with gamma making up the majority of the value) and delta tocopherols compared to other samples. There were three outliers that were not included in Figure 4.6 (early season MX grade 1 whole and early/mid season MX both grade 4 whole and mesocarp samples). Because these results were analyzed with a diode array detector it is suspected that these samples contained another compound that falsely elevated their tocopherol content. ANOVA further elucidated this data, with each tocopherol contributing to significant differences (alpha <0.01) between whole fruit and mesocarp oils. However, it was also found that gamma/beta tocopherols contributed to significant differences between grades. Most, if not all of literature, has quantified the tocopherols in avocado oil made from only the mesocarp. Our mesocarp only oil samples are

comparable to literature, which reports that oil made from Hass fruits contain mostly alpha tocopherol (Fernandes et al. 2018; Jorge et al. 2015; Lu et al. 2004). However, this trend is not consistent for oils made from the whole fruit.

These results are opposite of FAP and sterols, where timepoint and region dominated clustering. This has important implications for avocado oil standards — tocopherols may not be reliably used as a purity parameter for avocado oil because they change significantly depending on whole fruit/mesocarp oil and fruit grade. Because avocado oil can be made from whole or mesocarp of avocados with varying quality grades, it will be necessary to broaden the ranges of tocopherols in the standards which may decrease its utility for differentiating authentic avocado oil from adulterated ones.

Conclusions

This was the first study to analyze purity parameters of a comprehensive set of authentic samples for the purposes of supporting standard development. Samples overall fit the current proposed CODEX standards for fatty acid profile. Fruit growing region and harvest time were found to have the biggest impact on fatty acid profile and will be important to consider in the future as more regions become avocado oil producers. Knowing that fatty acids are affected by temperature, climate change is another variable to consider and studies with harvests spanning several years would be valuable to see the range of natural variances' impacts. This study has the most complete set of avocado oil sterols to date and although continued research is needed, we showed sterols were also impacted by region and harvest time but to a lesser extent than fatty acids. Standards for FAP and sterols do not need to be adjusted to accommodate oils made from the whole fruit vs only the mesocarp or for grade 1 vs grade 4 fruits. This can potentially help with food waste and waste produced from avocado oil production if poorer quality whole fruits

can be used to make oil rather than being thrown out completely or discarding the seed and exocarp. Tocopherols saw significantly differences in whole fruit oil and mesocarp oil. This is the first time this has been reported and the inclusion of tocopherols as a purity parameter should be carefully considered and limits to be adjusted for natural variances and processing technologies.

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Competing Interests

The authors have no competing interests to declare.

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Tables

Table 4.1. Fatty acid ranges seen across 68 samples in this study compared to current CODEX proposed standards.

Fatty Acid	Range in samples (% of total fatty acids)	Proposed CODEX Standards (% of total fatty acids)	Number of samples that do not pass current proposed standards (% of total samples)
C14:0	0.03-0.11	ND-0.3	0
C16:0	9.90-23.19	11.0-26.0	1.5
C16:1	3.32-10.03	4.0-17.1	2.9
C17:0	ND-0.09	ND-0.3	0
C17:1	0.08-0.11	ND-0.1	5.9
C18:0	0.29-0.68	0.1-1.3	0
C18:1	49.74-71.41	42.0-75.0	0
C18:2	9.25-22.46	7.8-19.0	5.9
C18:3	0.94-1.53	0.5-2.1	0
C20:0	0.08-0.17	ND-0.7	0
C20:1	0.17-0.23	ND-0.3	0
C22:0	ND-0.08	ND-0.5	0
C24:0	ND	ND-0.2	0

Table 4.2. Sterol ranges seen across 68 samples in this study compared to CODEX proposed standards. Numbers in brackets are values that have been proposed to CODEX but limits have not yet been agreed upon.

Sterol	Range in samples (% of total sterols)	Proposed CODEX standards (% of total sterols)	Number of samples that do not pass current proposed standards (% of total samples)
Cholesterol	ND	ND-0.5	0
Brassicasterol	ND	ND-0.5	0
Campesterol	4.85-9.75	4.0-8.3	11.8
Stigmasterol	ND-1.36	0.3-2.0	33.8
Clerosterol	1.47-3.49	[0.6] [1.0]-2.0	39.7
Beta-sitosterol	81.71-85.87	[71.0] [79.0]-93.4	0
Delta-5-avenasterol	3.80-8.45	2.0-8.0	4.4
Delta-7-stigmastenol	ND-0.09	ND-[1.0] [3.5]	0
Delta-7-avenasterol	ND-0.27	ND-1.5	0

Table 4.3. Tocopherols±SD from experimental design one. Gamma and beta tocopherols were quantified together. Letters show significant differences between samples using the Tukey test; samples with a star were not included due to being outliers.

Region	Harvest time	Grade	Processing	Alpha (mg/kg)	Gamma & beta (mg/kg)	Delta (mg/kg)	Total tocopherols (mg/kg)
CA	Early 1	Grade 1	Whole	135±25.2^{abcdef}	15.5±5^{cdef}	33.4±5.8^{b}	183.9±35.7^{abc}
			Mesocarp	101.4±15.5^{cdefgh}	ND	ND	101.4±15.5^{ghij}
		Grade 4	Whole	152.2±22^{abc}	ND	48.6±12.5^{a}	200.7±34.5^{a}
			Mesocarp	131.5±18.2^{abcdef}	ND	ND	131.5±18.2^{cdefghi}
	Mid 3	Grade 1	Whole	158.8±16.8^{ab}	23.4±4.7^{abc}	19.6±2.6^{efg}	201.8±21.6^{a}
			Mesocarp	112.2±10.2^{abcdefgh}	ND	ND	112.2±10.2^{fghij}
		Grade 4	Whole	147.4±10.7^{abcd}	ND	22.8±1.4^{cde}	170.2±11.7^{abcdef}
			Mesocarp	120.2±7.8^{abcdefgh}	ND	ND	120.2±7.7^{defghij}
	Late 5	Grade 1	Whole	129.9±1.8^{abcdef}	29.7±8.4^{a}	26.4±2.5^{bcde}	186.1±8.7^{abc}
			Mesocarp	94.9±7.8^{cdefgh}	ND	ND	94.9±7.7^{ghij}
		Grade 4	Whole	149±14.3^{abcd}	ND	29.2±3.3^{bcd}	178.2±11.3^{abcd}
			Mesocarp	101.6±10.6^{efgh}	ND	ND	101.6±10.6^{ghij}
MX	Early 1	Grade 1	Whole	199.1±49.1*	24.9±2.3*	47.7±1.3*	271.7±50.6*
			Mesocarp	117.6±16.4^{abcdefgh}	ND	ND	117.6±16.4^{efghij}
		Grade 4	Whole	162.4±46.5^{a}	ND	31.6±4^{bc}	194±46.5^{ab}
			Mesocarp	141.5±8.6^{abcdef}	ND	ND	141.5±8.6^{bcdefgh}
	Early/mid 2	Grade 1	Whole	134.1±8.5^{abcdef}	22±2.1^{abcd}	25.4±1.9^{bcde}	181.4±12.1^{abc}
			Mesocarp	71.1±3.4^{h}	6.3±1.4^{gh}	ND	77.4±4.5^{ij}
		Grade 4	Whole	115.2±4.5*	197.8±11.2*	ND	312.9±15*
			Mesocarp	77.1±10.3*	61.9±18.4*	ND	138.9±28.6*
	Mid 3	Grade 1	Whole	127.4±6.7^{abcdefg}	22.2±1.8^{abcd}	25.4±1.9^{bcde}	175±6.6^{abcde}
			Mesocarp	107.7±11.3^{bcdefgh}	10.9±2.8^{fg}	ND	118.7±13.3^{efghij}
		Grade 4	Whole	116.6±23.4^{abcdefgh}	26±6.1^{ab}	10.2±1.8^{g}	152.8±29.8^{abcdefg}
			Mesocarp	90.7±11.5^{fgh}	12.8±1.6^{efg}	ND	103.6±12.7^{ghij}
	Mid/late 4	Grade 1	Whole	143.8±16^{abcde}	19.3±1.4^{bcde}	33±2.8^{b}	196.2±20^{ab}
			Mesocarp	91.5±10.8^{fgh}	ND	ND	91.5±10.8^{hij}
		Grade 4	Whole	101.1±15.1^{cdefgh}	ND	11±1.2^{g}	112.2±16.3^{fghij}
			Mesocarp	71.2±9.7^{h}	ND	ND	71.2±9.7^{j}
	Late 5	Grade 1	Whole	144.3±12^{abcde}	14.3±1.3^{defg}	21±1.9^{def}	179.5±15.2^{abc}
			Mesocarp	108.7±10.6^{bcdefgh}	ND	ND	108.7±10.6^{ghij}
		Grade 4	Whole	98.8±16.1^{cdefgh}	ND	13.2±2.2^{fg}	112±18.3^{fghij}
			Mesocarp	76.8±10.1^{gh}	ND	ND	76.8±10.1^{ij}

Figures

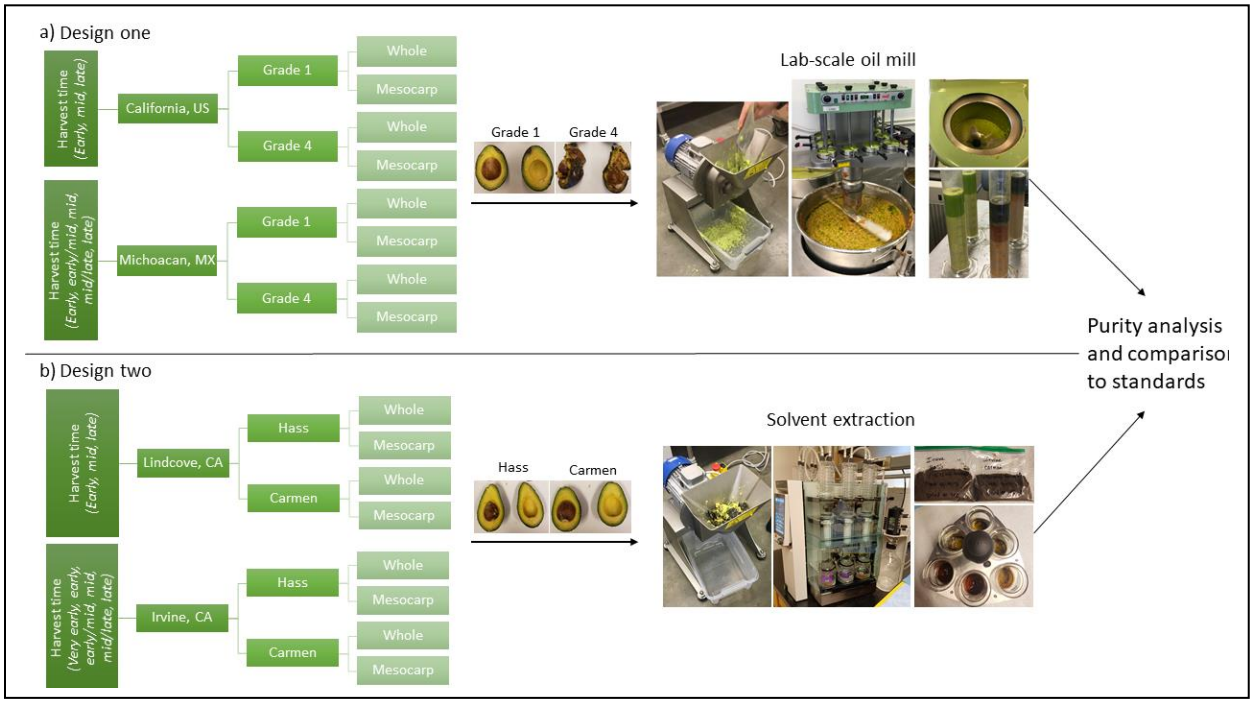


Figure 4.1. Flow chart showing extraction process for experimental designs one (a) and design two (b).

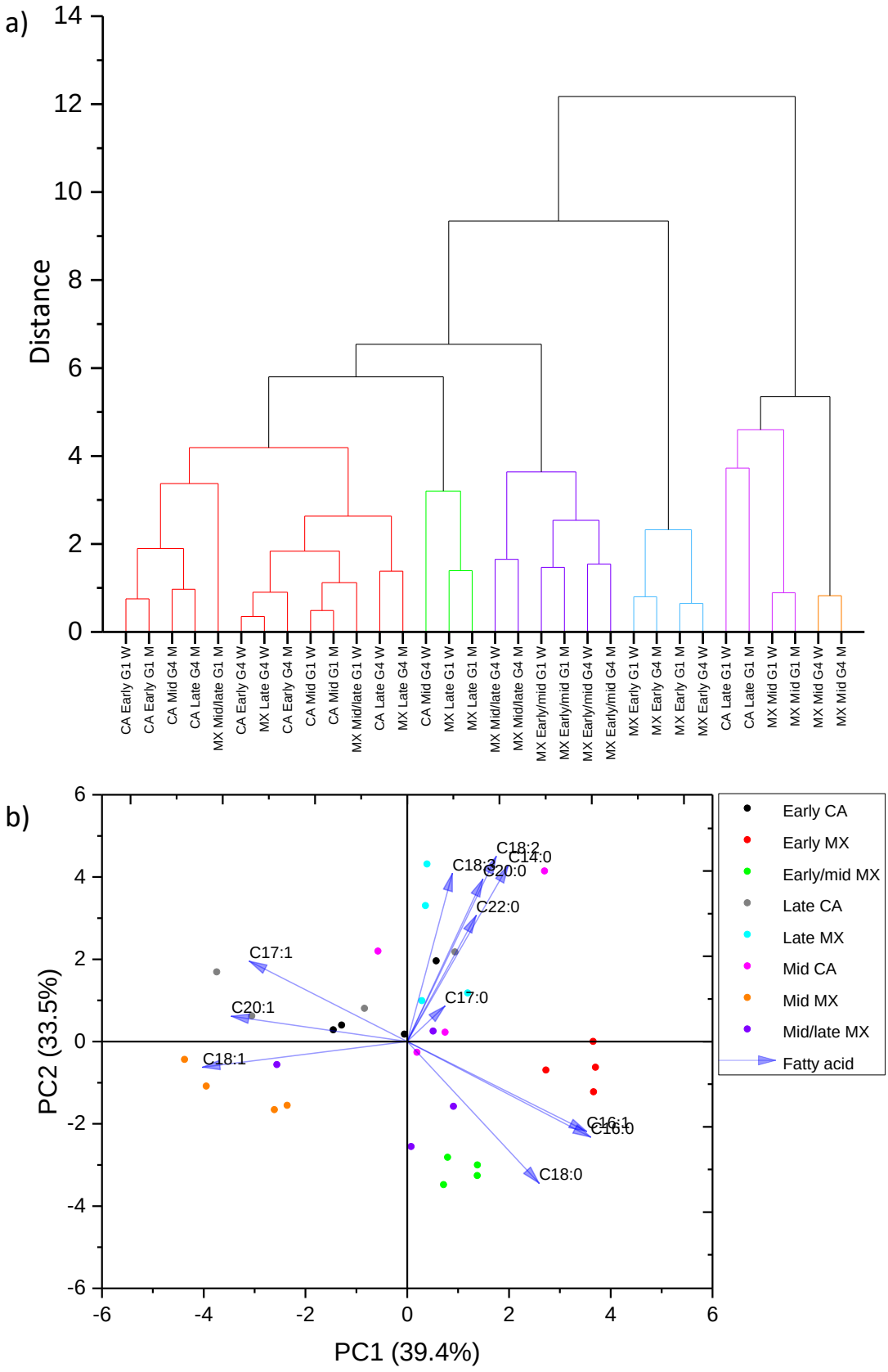

a)
Distance
14
12
10
8
6
4
2
0
CA Early G1 W
CA Early G1 M
CA Mid G4 M
CA Late G4 M
MX Mid/late G1 M
CA Early G4 W
MX Late G4 W
CA Early G4 M
CA Mid G1 W
CA Mid G1 M
MX Mid/late G1 W
CA Late G4 W
MX Late G4 M
CA Mid G4 W
MX Late G1 W
MX Late G1 M
MX Mid/late G4 W
MX Mid/late G4 M
MX Early/mid G1 W
MX Early/mid G1 M
MX Early/mid G4 W
MX Early/mid G4 M
MX Early G1 W
MX Early G4 M
MX Early G1 M
MX Early G4 W
CA Late G1 W
CA Late G1 M
MX Mid G1 W
MX Mid G1 M
MX Mid G4 W
MX Mid G4 M
b)
PC2 (33.5%)
PC1 (39.4%)
6
4
2
0
-2
-4
-6
C18:2
C14:0
C18:3
C20:0
C22:0
C17:1
C17:0
C20:1
C18:1
C16:1
C16:0
C18:0
Early CA
Early MX
Early/mid MX
Late CA
Late MX
Mid CA
Mid MX
Mid/late MX
Fatty acid

Figure 4.2. a) Hierarchal cluster analysis was used to organize samples from experimental design one based on their fatty acid profile. b) A PCA was performed on the same data set, which accounted for 72.9% of variance. Samples were labeled according to region and timepoint, the two main variables contributing to clustering in “a”.

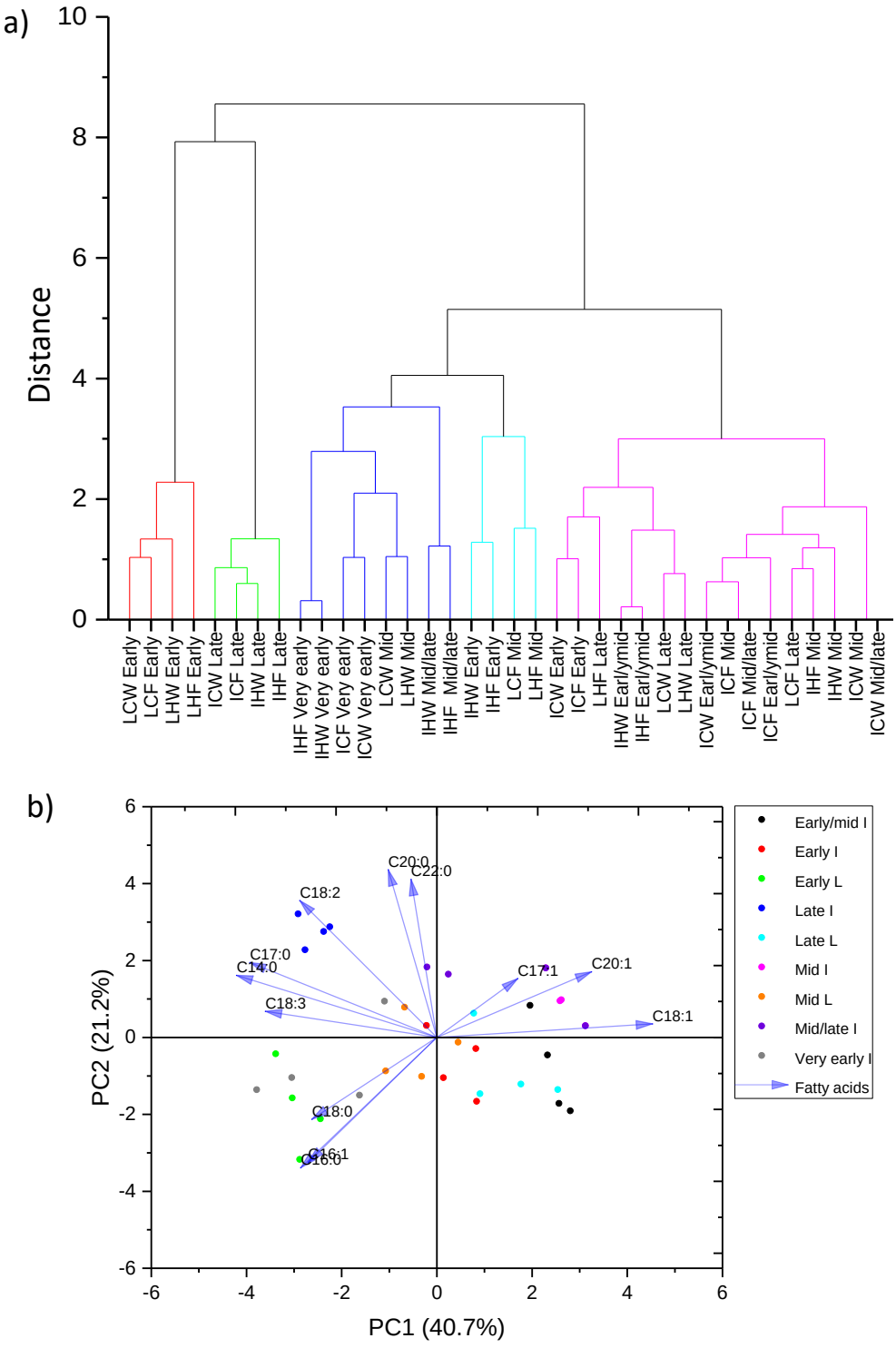
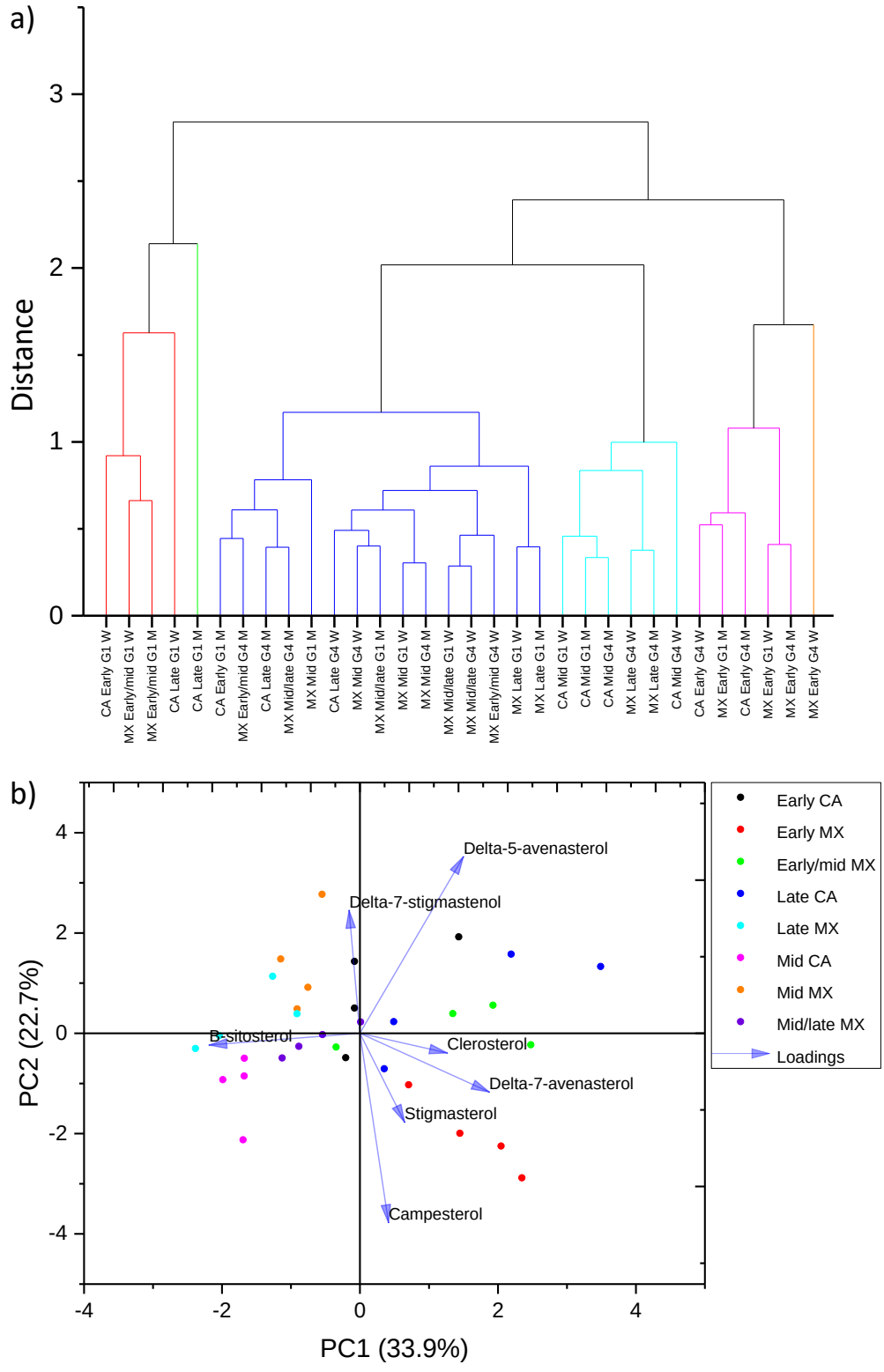


Figure 4.3. a) Hierarchal cluster analysis was used to display design two samples using their fatty acid profile. L stands for Lindcove, I for Irvine, H for Hass, C for Carmen, W for whole and M for Mesocarp. b) A PCA was performed on the same data set, which accounted for 68.9% of

variance. Samples were labeled according to region and timepoint, the two main variables contributing to clustering in “a”.



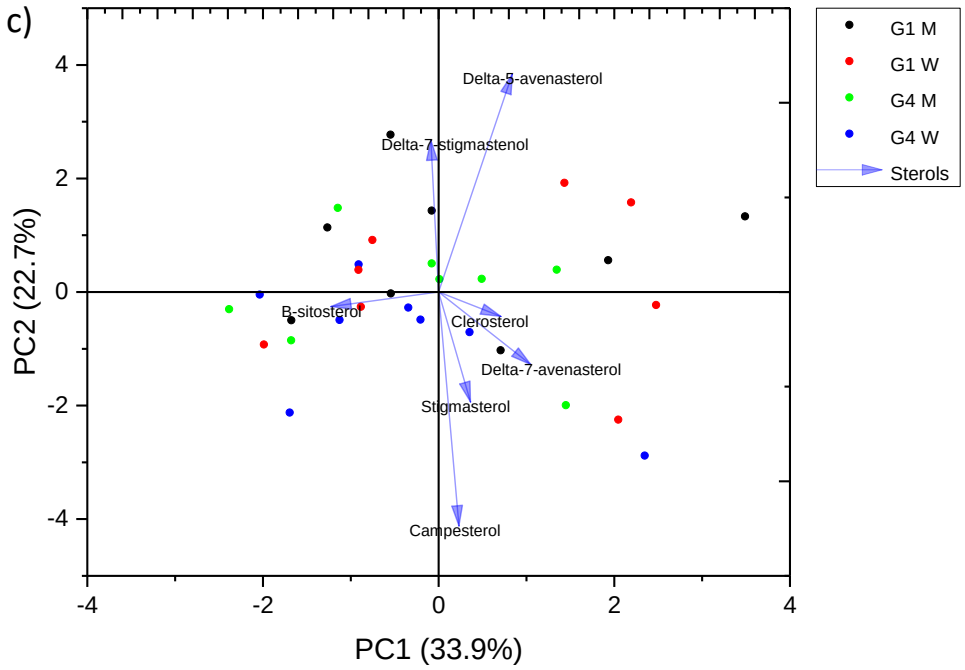


Figure 4.4. a) Hierarchal cluster analysis of samples based on sterols profile. b) PCA of samples with sterols as treatments. Samples labeled according to region and harvest time. c) Same PCA as “b” with samples labeled according to grade (G1 or G4) and processing (M for mesocarp and W for whole).

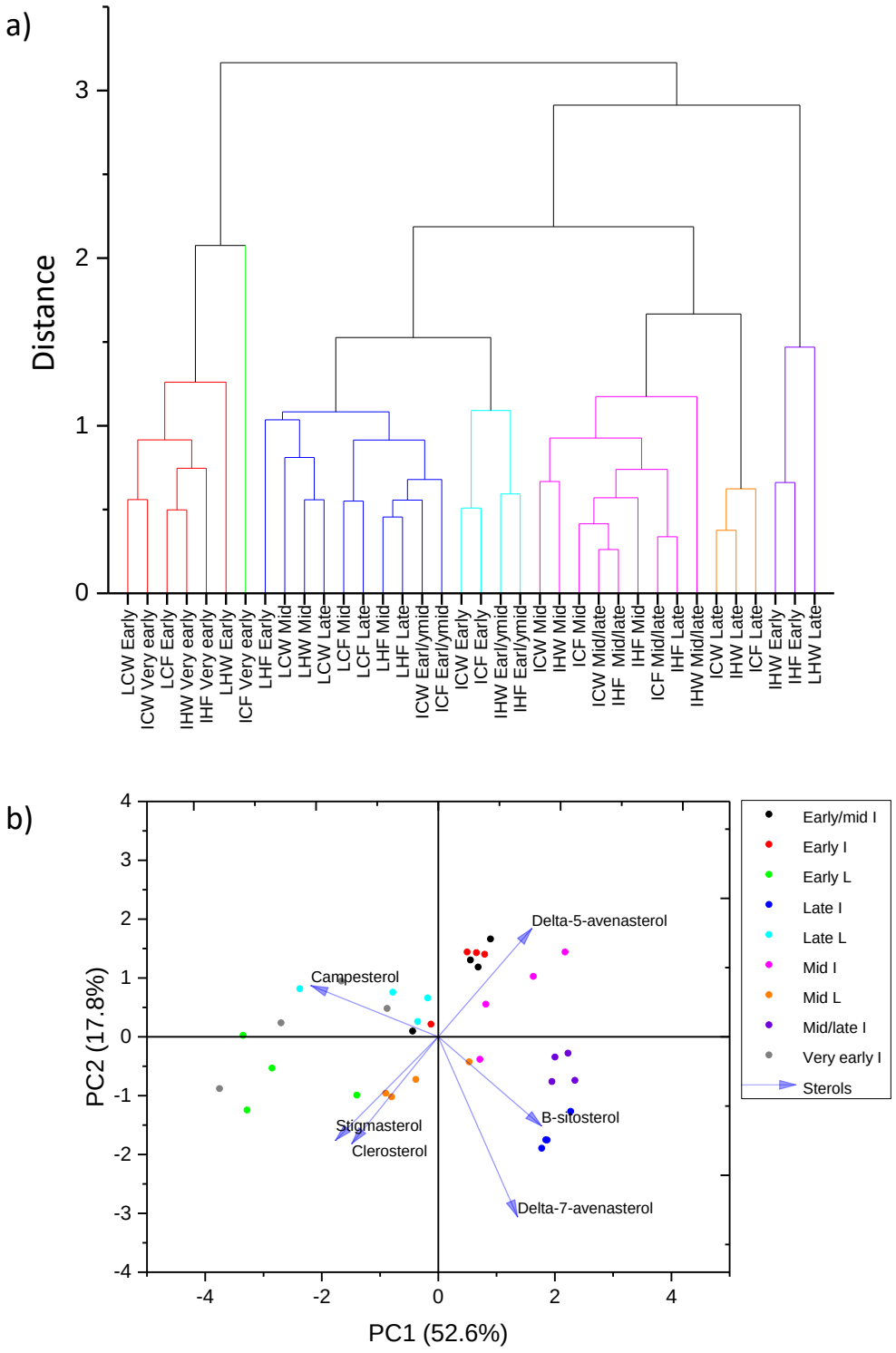


Figure 4.5. Multivariate analysis for sterols profile of design two samples. L stands for Lindcove, I for Irvine, H for Hass, C for Carmen, W for whole and M for Mesocarp a) Hierarchal cluster analysis. b) PCA plot with samples labeled according to region and harvest time.

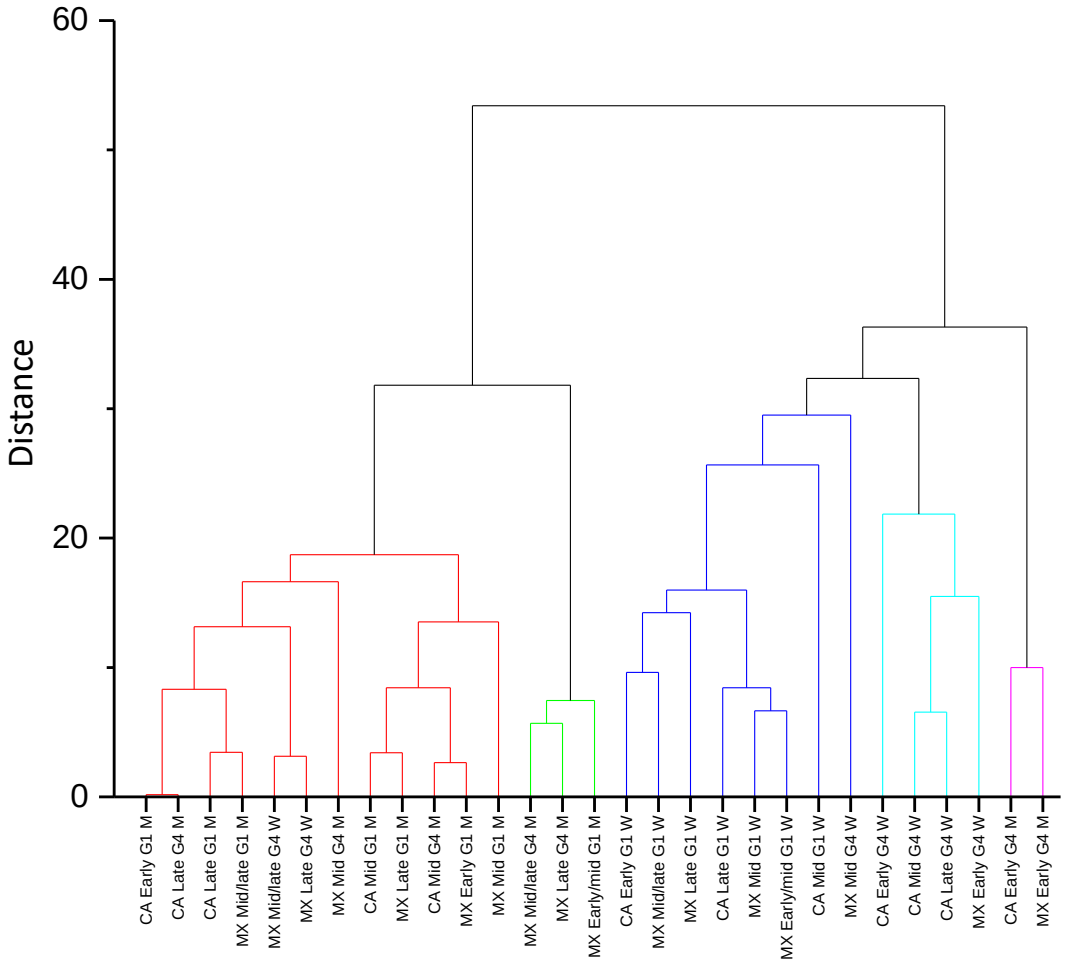


Figure 4.6. Hierarchal cluster analysis for tocopherol content for design one samples where G stands for grade, M for mesocarp, and W for whole.

Supplementary Tables

Table S4.1. Fatty acids dataset for design one, California samples reported as %±SD. The first column is harvest time, followed by grade 1 or 4, and processing where M is for mesocarp and W for whole. Samples in red did not meet proposed standards

			C14:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0
Early	G1	W	0.04±0	15.36±0.01	6.48±0	0.04±0.02	0.1±0.01	0.41±0	62.91±0.02	13.46±0.01	0.86±0	0.11±0	0.19±0	0.03±0
		M	0.04±0	15.99±0.01	6.27±0	0.03±0	0.11±0	0.43±0	62.6±0.01	13.31±0	0.89±0	0.1±0	0.19±0	0.03±0.01
	G4	W	0.05±0	16.37±0.04	6.65±0	0.03±0	0.1±0	0.47±0	60.15±0.02	14.8±0.01	1±0	0.12±0	0.18±0	0.08±0.01
		M	0.05±0	16.62±0.02	6.8±0	0.03±0	0.1±0	0.47±0	60.56±0.01	14.17±0.01	0.89±0	0.1±0	0.18±0	0.03±0
Mid	G1	W	0.05±0	17.57±0.14	7.34±0.02	0±0.03	0.09±0	0.48±0	59.66±0.1	13.62±0.05	0.82±0	0.13±0	0.18±0	0.04±0.01
		M	0.05±0	17.48±0.01	7.13±0	0±0.04	0.09±0	0.47±0	60.08±0.04	13.53±0.01	0.81±0	0.11±0.01	0.18±0	0.04±0
	G4	W	0.08±0	17.5±0.04	6.5±0.01	0±0	0.09±0	0.47±0	56.11±0.02	17.74±0	1.1±0	0.16±0	0.17±0	0.08±0.05
		M	0.06±0	14.62±0.01	5.8±0	0.07±0	0.11±0	0.46±0	62.48±0.02	15.05±0	1.01±0	0.12±0.01	0.19±0	0.04±0
Late	G1	W	0.04±0	9.9±3.85	4.63±0.2	0±0.02	0.11±0	0.41±0.02	70.38±3.02	13.21±0.56	0.95±0.04	0.12±0	0.21±0.01	0.04±0.01
		M	0.04±0	12.8±0.01	4.61±0.01	0±0.02	0.1±0	0.41±0	68.1±0.04	12.65±0	0.93±0	0.1±0	0.2±0	0.04±0.01
	G4	W	0.06±0	15.75±0.27	6.64±0.12	0.04±0.02	0.1±0	0.47±0.01	58.78±0.68	16.79±0.29	1.03±0.02	0.13±0	0.17±0	0.04±0.01
		M	0.05±0	15.33±0.04	6.09±0.01	0±0.03	0.11±0	0.45±0	61.96±0.01	14.77±0.01	0.9±0	0.1±0	0.18±0	0.04±0

Table S4.2. Fatty acids dataset for design one, Mexico samples reported as %±SD. The first column is harvest time, followed by grade 1 or 4, and processing where M is for mesocarp and W for whole. Samples in red did not meet proposed standards

			C14:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0
Early	G1	W	0.05 ±0	21.47± 0.01	9.86± 0.01	0.03± 0	0.08± 0	0.57± 0	52.5± 0.01	14.18 ±0	0.96± 0	0.1±0	0.17± 0	0.04± 0.01
		M	0.05 ±0	19.93± 0.02	9.21± 0	0.03± 0	0.09± 0	0.54± 0	53.61 ±0.02	15.19 ±0	1.07± 0.01	0.1±0	0.17± 0	0.02± 0.01
	G4	W	0.06 ±0	19.98± 0	8.63± 0.01	0.03± 0	0.08± 0	0.63± 0	53.8± 0.01	15.21 ±0.01	1.26± 0	0.11± 0	0.18± 0	0.04± 0.01
		M	0.06 ±0	21.25± 0.04	9.67± 0	0.03± 0	0.09± 0	0.59± 0	52.11 ±0.03	14.79 ±0.01	1.12± 0	0.1±0	0.17± 0	0.03± 0
Early /mid	G1	W	0.03 ±0	19.28± 0.03	8.17± 0	0.02± 0.02	0.08± 0.01	0.62± 0.02	61.52 ±0.02	9.27± 0.01	0.68± 0	0.1± 0.01	0.17± 0.01	0.04± 0.02
		M	0.03 ±0	19.92± 0	8.77± 0	0.01± 0	0.09± 0.01	0.63± 0	60.34 ±0.07	9.25± 0	0.64± 0	0.1±0	0.17± 0	0.04± 0.06
	G4	W	0.04 ±0	19.16± 0.29	8.75± 1.47	0.01± 0	0.09± 0.01	0.63± 0.01	59.52 ±0.96	10.76 ±0.18	0.73± 0.01	0.11± 0	0.19± 0.01	0.01± 0.01
		M	0.03 ±0	19.21± 0	10.03 ±0.01	0±0.0 1	0.09± 0.01	0.58± 0.01	58.85 ±0.01	10.25 ±0	0.67± 0	0.1± 0.01	0.18± 0.01	ND
Mid	G1	W	0.04 ±0	13.15± 0	3.32± 0	0±0.0 1	0.1±0	0.47± 0	71.41 ±0.01	10.16 ±0.01	1±0	0.09± 0	0.23± 0	0.02± 0
		M	0.04 ±0	13.48± 0.02	3.91± 0	0.03± 0	0.1±0	0.47± 0	71±0. 02	9.77± 0.02	0.87± 0.01	0.08± 0	0.22± 0	0.02± 0
	G4	W	0.03 ±0	15.81± 0	5.31± 0	0.02± 0.01	0.1±0	0.51± 0	67.01 ±0.01	10.05 ±0	0.83± 0	0.1±0	0.21± 0	0.02± 0
		M	0.04 ±0	15.68± 0.01	5.24± 0	0.02± 0.01	0.1±0	0.49± 0	67.69 ±0.01	9.61± 0	0.8±0	0.09± 0	0.21± 0	0.02± 0
Mid/ late	G1	W	0.05 ±0	17.65± 1.24	7.17± 0.89	0.02± 0	0.09± 0	0.49± 0.03	58.98 ±2.11	14.2± 0.01	1.02± 0.02	0.12± 0	0.2± 0.01	0.03± 0
		M	0.04 ±0	14.79± 0.01	6.06± 0	0.02± 0	0.1±0	0.42± 0	65.1± 0	12.25 ±0	0.91± 0.01	0.1±0	0.22± 0	ND
	G4	W	0.05 ±0	18.2± 0.07	7.25± 0.01	0.01± 0	0.09± 0	0.58± 0	61.24 ±0.05	11.53 ±0.02	0.75± 0	0.1±0	0.18± 0	0.03± 0.01
		M	0.04 ±0	18± 0.04	6.58± 0.01	0.01± 0	0.08± 0	0.55± 0	62.67 ±0.02	11.07 ±0	0.72± 0.01	0.09± 0	0.18± 0	0.02± 0.01
Late	G1	W	0.07 ±0	15.34± 0.1	5.42± 0.03	0.02± 0	0.1±0	0.45± 0	57.59 ±0.27	19.09 ±0.43	1.53± 0.01	0.14± 0	0.22± 0.02	0.03± 0
		M	0.06 ±0	15.51± 0.01	5.57± 0.01	0.02± 0	0.09± 0.01	0.45± 0	58.47 ±0.06	18.04 ±0.02	1.42± 0.01	0.12± 0	0.2±0	0.04± 0.01
	G4	W	0.05 ±0	16.38± 0.01	7±0	0.02± 0	0.08± 0	0.49± 0	58.88 ±0	15.62 ±0.01	1.12± 0	0.13± 0	0.19± 0	0.04± 0
		M	0.05 ±0	16.08± 0	6.79± 0.01	0.02± 0	0.1±0	0.48± 0	60.19 ±0	14.91 ±0	1.05± 0	0.11± 0	0.18± 0	0.04± 0.01

Table S4.3. Fatty acids reported as %±SD for design two, Lindcove samples. The first column is harvest time, followed by cultivar (Car = Carmen), and processing where M is for mesocarp, and W is for whole. All samples met proposed standards.

			C14:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0
Early	Car	W	0.09± 0	22.04 ±0.37	8.6± 0.34	0.08± 0	0.09± 0.01	0.56± 0.01	49.74 ±0.68	17.24 ± 0.57	1.24± 0.09	0.1± 0.01	0.17± 0.01	0.05± 0.01
		M	0.1±0	21.47 ±0.52	8.89± 0.39	0.08± 0.04	0.09± 0.01	0.56± 0.01	50.47 ±0.73	16.89 ± 0.53	1.17± 0.09	0.11± 0	0.17± 0.01	0±0.0 4
	Hass	W	0.08± 0	22.27 ±0.07	8.59± 0.06	0.04± 0.06	0.09± 0	0.59± 0	50.75 ±0.06	16.14 ± 0.04	1.19± 0.01	0.07± 0.07	0.16± 0	0.03± 0.01
		M	0.07± 0.01	23.19 ±0.28	9.67± 0.09	0.05± 0.03	0.09± 0	0.6±0	49.21 ±0.1	15.82 ± 0.07	1.04± 0	0.1± 0.01	0.15± 0	0.01± 0.02
Mid	Car	W	0.07± 0	19.04 ±1.00	6.68± 0.22	0.04± 0.02	0.09± 0	0.54± 0.02	54.92 ±0.01	16.96 ±0 .94	1.3± 0.16	0.11± 0	0.19± 0	0.06± 0.01
		M	0.07± 0.03	20.9± 4.52	6.65± 0.25	0.05± 0.02	0.1±0. 01	0.6± 0.08	55.98 ±0.01	14.28 ± 3.64	1.01± 0.32	0.1± 0.01	0.21± 0.03	0.05± 0.01
	Hass	W	0.07± 0.01	19.37 ±1.43	7.24± 0.85	0.05± 0.03	0.09± 0	0.64± 0.05	54.84 ±0.04	16.14 ± 1.67	1.25± 0.26	0.11± 0	0.17± 0.01	0.02± 0.04
		M	0.07± 0.01	21.09 ±3.02	7.36± 0.67	0.04± 0.04	0.1± 0.01	0.68± 0.1	54.69 ±0.11	14.57 ± 3.08	1.08± 0.35	0.1± 0.01	0.17± 0.01	0.04± 0.04
Late	Car	W	0.06± 0	16.7± 0.61	7.2± 0.4	ND	0.09± 0.01	0.48± 0.02	57.29 ±0.04	16.71 ± 0.97	1.23± 0.11	0.02± 0.04	0.21± 0	0.01± 0.01
		M	0.05± 0.01	16.85 ±0.85	7.08± 0.11	0.01± 0.02	0.09± 0.01	0.47± 0.01	58.64 ±0.02	15.48 ± 1.02	1.07± 0.14	0.02± 0.03	0.22± 0.02	0.02± 0
	Hass	W	0.06± 0.01	17.16 ±0.59	6.94± 0.78	0.04± 0.04	0.09± 0	0.49± 0.03	56.77 ±0.11	16.85 ± 1.51	1.27± 0.18	0.1± 0.03	0.2±0	0.04± 0.01
		M	0.06± 0.01	18.33 ±2.25	7.61± 1.04	0.03± 0.03	0.09± 0.02	0.52± 0.09	57.13 ±0.02	14.88 ± 2.48	1.09± 0.29	0.05± 0.05	0.19± 0.01	0.02± 0.02

Table S4.4. Fatty acids reported as %±SD for design two, Irvine samples. The first column is harvest time, followed by cultivar (Car = Carmen), and processing where M is mesocarp and W is whole. Samples in red did not meet proposed standards.

			C14:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0
Very Early	Car	W	0.11±0	18.92±0.18	6.88±0.07	0.07±0.03	0.09±0.01	0.59±0	53.96±0.07	17.81±0.04	1.42±0.01	0.07±0.09	0.08±0.12	0.01±0.01
		M	0.09±0.01	18.65±0.03	6.66±0.08	0.06±0.02	0.1±0	0.29±0.38	53.77±0.28	18.71±0.12	1.43±0.01	0.07±0.06	0.16±0	0.02±0.03
	Hass	W	0.1±0	19.62±0.01	8.22±0.08	0.08±0.02	0.1±0	0.64±0.01	52.29±0.02	17.4±0.06	1.32±0.01	0.08±0.08	0.15±0	0.01±0.02
		M	0.07±0	19.43±0.54	8.43±0.11	0.04±0.05	0.1±0	0.59±0.01	52.39±0.32	17.39±0.13	1.33±0.01	0.06±0.05	0.15±0	0.02±0.02
Early	Car	W	0.09±0.01	18.96±0.36	6.59±0.66	0.02±0.02	0.09±0	0.54±0.03	56.01±0.01	16.27±1.38	1.11±0.01	0.11±0	0.17±0.01	0.05±0.03
		M	0.07±0.01	18.66±0.9	6.81±0.86	0.03±0.02	0.09±0	0.49±0	56.77±0.01	15.72±0.13	1.05±0.1	0.09±0	0.18±0	0.03±0.01
	Hass	W	0.07±0.01	18.52±0.59	8.97±0.7	0.04±0.02	0.1±0.01	0.49±0.01	54.84±0.04	15.65±0.32	1.03±0.09	0.11±0.01	0.17±0.01	0.01±0.03
		M	0.05±0	19.09±0.31	8.4±0.6	0.04±0.04	0.1±0	0.49±0.03	55.59±0.03	15±0.74	0.96±0.02	0.09±0	0.18±0.01	0.01±0.01
Early /mid	Car	W	0.06±0	17.15±0.16	5.94±0.34	0.01±0.02	0.09±0.01	0.52±0	58.97±0	15.83±0.57	1.07±0.02	0.1±0.05	0.2±0	0.06±0.04
		M	0.06±0	17.74±0.45	6.17±0.64	ND	0.1±0.01	0.52±0.01	58.73±0.13	15.49±1.38	1±0.02	0±0	0.19±0.01	ND
	Hass	W	0.06±0	17.19±0.59	7.29±0.36	ND	0.1±0.01	0.47±0.01	57.81±0.01	15.74±0.49	1.06±0.02	0.04±0.08	0.2±0.01	0.04±0.04
		M	0.06±0.01	17.2±0.7	7.43±0.52	ND	0.1±0.02	0.47±0.04	57.88±0.03	15.61±2.22	1.04±0.2	0.02±0.03	0.19±0.01	ND
Mid	Car	W	0.07±0	17.16±0.21	5.55±0.21	0.05±0	0.1±0	0.46±0	60.45±0.01	14.8±0.7	0.96±0.02	0.15±0.02	0.21±0.01	0.04±0.01
		M	0.04±0.02	16.98±0.04	5.94±0.37	0.01±0.02	0.1±0	0.44±0.01	59.54±0.12	15.68±1.3	0.94±0.06	0.12±0.03	0.18±0.01	0.02±0.03
	Hass	W	0.06±0.01	16.48±0.75	6.58±0.09	0.02±0.02	0.1±0	0.42±0.01	59.74±0.01	15.26±1.3	0.96±0.09	0.15±0.02	0.19±0	0.02±0.02
		M	0.06±0.01	16.17±0.81	7.13±0.27	0.02±0.02	0.1±0	0.42±0.03	59.02±0.01	15.76±1.31	0.98±0.12	0.12±0.01	0.19±0	0.03±0.01
Mid/ late	Car	W	0.07±0	17.16±0.21	5.55±0.21	0.05±0	0.1±0	0.46±0	60.45±0.06	14.8±0.7	0.96±0.02	0.15±0.02	0.21±0.01	0.04±0.01
		M	0.04±0.02	16.98±0.04	5.94±0.37	0.01±0.02	0.1±0	0.44±0.01	59.54±0.01	15.68±1.3	0.94±0.06	0.12±0.03	0.18±0.01	0.02±0.03
	Hass	W	0.07±0.01	16.79±0.14	7.31±0.26	0.05±0	0.1±0	0.47±0.02	55.73±0.09	18.05±1.11	1.06±0.05	0.15±0	0.18±0	0.04±0
		M	0.07±0.01	16.55±0.61	7.31±0.04	0.06±0.01	0.1±0	0.48±0.03	54.97±0.01	18.97±1.25	1.12±0.1	0.13±0.01	0.18±0.01	0.05±0
Late	Car	W	0.1±0.01	17.45±0.22	6.53±0.25	0.06±0	0.09±0	0.55±0.03	51.6±0.03	21.95±1.26	1.25±0.07	0.17±0.02	0.19±0	0.05±0
		M	0.1±0.01	17.24±1.08	6.75±0.23	0.07±0.01	0.1±0	0.52±0.03	51.15±0.04	22.46±1.45	1.25±0.13	0.13±0	0.19±0	0.05±0
	Hass	W	0.1±0	17.12±0.48	7.21±0.02	0.09±0.02	0.1±0	0.53±0.01	51.02±0.02	22.12±3.16	1.32±0.28	0.16±0.02	0.18±0.01	0.05±0.01
		M	0.09±0	17.26±0.55	7.66±0.06	0.07±0.01	0.09±0	0.51±0.02	50.26±0.04	22.4±1.65	1.3±0.17	0.14±0.01	0.17±0	0.05±0

Table S4.5. Sterols reported as %±SD for design one, California location. Each sterol name is abbreviated; all end with -sterol except delta-7-stigmastenol. The first column is harvest time, followed by grade 1 or 4, and processing, where M is mesocarp and W is whole. Samples in red did not meet proposed standards.

			Chole	Bras-sica	Campe	Stigma	Clero	B-sito	Δ-5-avena	Δ-7-stigma	Δ-7-avena
Early	G1	W	ND	ND	5.9± 0.03	0.22± 0.01	1.64± 0.03	83.57± 0.2	8.35± 0.21	0.24± 0.07	0.09± 0.12
		M	ND	ND	5.97± 0.09	0.18±0	1.71± 0.07	84.61± 0.26	7.26± 0.15	0.27± 0.08	ND
	G4	W	ND	ND	6.85± 0.06	0.41± 0.01	1.73± 0.06	84.64± 0.01	6.1± 0.03	0.28± 0	ND
		M	ND	ND	6.82± 0.02	0.28±0	1.68± 0.01	84.3± 0.03	6.51± 0.05	0.41± 0.04	ND
Mid	G1	W	ND	ND	6.42± 0.02	ND	1.88± 0.09	86.5±0.1	5.2± 0.01	ND	ND
		M	ND	ND	6.29± 0.01	ND	1.84± 0.01	86.21± 0.04	5.66± 0.04	ND	ND
	G4	W	ND	ND	6.27± 0.1	0.56±0	1.79±0	86.6± 0.05	4.78± 0.04	ND	ND
		M	ND	ND	6.48± 0.02	ND	1.92± 0.19	86.2± 0.14	5.4± 0.04	ND	ND
Late	G1	W	ND	ND	4.85± 0.11	0.28± 0.01	1.8± 0.04	84.34± 0.34	8.45± 0.17	ND	0.29± 0.01
		M	ND	ND	5.04± 0.35	0.2± 0.06	3.49± 2.52	83.13± 2.38	8.02± 0.41	ND	0.1± 0.15
	G4	W	ND	ND	5.96± 0.04	0.45± 0.02	1.91± 0.11	85.14± 0.77	6.45± 0.46	ND	0.1± 0.14
		M	ND	ND	5.65± 0.02	0.25± 0.01	1.91± 0.07	85.08± 0.14	6.99± 0.12	ND	0.13± 0.06

Table S4.6. Sterols reported as %±SD for design one, Mexico location. Each sterol name is abbreviated; all end with -sterol except delta-7-stigmastenol. The first column is harvest time, followed by grade 1 or 4, and processing, where M is mesocarp and W is whole. Samples in red did not meet proposed standards.

			Chole	Bras-sica	Campe	Stigma	Clero	B-sito	Δ-5-avena	Δ-7-stigma	Δ-7-avena
Early	G1	W	ND	ND	7.37± 0.01	0.3± 0.01	1.89± 0.22	83.99± 0.65	6.12± 0.22	ND	0.33± 0.21
		M	ND	ND	6.77± 0.04	0.16± 0.01	1.9± 0.02	84.64± 0.13	6.37± 0.05	ND	0.16± 0.01
	G4	W	ND	ND	8.09± 0.03	0.32± 0.02	2.35± 0.96	83.16± 0.86	5.89± 0.04	ND	0.19± 0.01
		M	ND	ND	7.67± 0.04	0.2± 0.01	1.91± 0.01	83.8± 0.1	6.23± 0.09	ND	0.19± 0.01
Early / mid	G1	W	ND	ND	6.51± 0.11	0.2±0	1.95±0	83.48± 0.41	7.59± 0.27	ND	0.26± 0.02
		M	ND	ND	6.08± 0.01	0.08±0	1.88± 0.11	83.91± 0.53	7.81± 0.41	ND	0.24± 0.03
	G4	W	ND	ND	5.72± 0.03	0.35± 0.03	1.47± 0.04	85.77± 0.34	6.54± 0.3	ND	0.15± 0.01
		M	ND	ND	5.81± 0.02	0.14±0	1.88± 0.01	84.56± 0.12	7.37± 0.12	ND	0.23± 0.01
Mid	G1	W	ND	ND	5.84± 0.02	0.09± 0.13	1.7± 0.04	85.36± 0.48	6.91± 0.18	0.1± 0.14	ND
		M	ND	ND	5.39± 0.05	0±0	1.72± 0.14	85.03± 0.1	7.49± 0.02	0.38± 0.03	ND
	G4	W	ND	ND	5.94± 0.03	0.21± 0.01	1.68± 0.05	85.52± 0.14	6.5± 0.09	0.15± 0.21	ND
		M	ND	ND	5.72± 0.01	0±0	1.6± 0.02	85.59± 0.34	6.92± 0.06	0.18± 0.26	ND
Mid/ late	G1	W	ND	ND	5.82± 0.08	0.33±0	1.75± 0.02	85.77± 0.22	6.33± 0.12	ND	ND
		M	ND	ND	6.12± 0.04	0.21± 0.01	1.73± 0.01	85.26± 0.06	6.69± 0.03	ND	ND
	G4	W	ND	ND	5.98± 0.02	0.33±0	1.64± 0.01	85.89± 0.02	6.16± 0.02	ND	ND
		M	ND	ND	5.78± 0.03	0.21±0	1.71± 0.1	85.57± 0.32	6.58± 0.02	0.16± 0.23	ND
Late	G1	W	ND	ND	5.37± 0.03	0.3±0	1.76± 0.08	85.92± 0.05	6.65±0	ND	ND
		M	ND	ND	5.32± 0.02	ND	1.77± 0.08	86.06± 0.14	6.85± 0.04	ND	ND
	G4	W	ND	ND	5.77± 0.05	ND	1.83± 0.02	86.71± 0.09	5.69± 0.06	ND	ND
		M	ND	ND	5.9± 0.11	ND	1.72± 0.04	86.94± 0.57	5.45± 0.5	ND	ND

Table S4.7. Sterols dataset for experimental design two, Lindcove location. Samples reported as %±SD. Each sterol is abbreviated; all end with -sterol except for delta-7-stigmastenol. The first column is harvest time, followed by cultivar (Car = Carmen) and processing, where M is mesocarp and W is whole. Samples in red did not meet proposed standards.

			Chole	Bras-sica	Campe	Stigma	Clero	B-sito	Δ-5-avena	Δ-7-stigma	Δ-7-avena
Early	Car	W	ND	ND	9.1± 0.03	1.36± 0.07	2.5± 0.44	82.87 ± 0.28	4.1± 0.3	ND	0.07± 0.1
		M	ND	ND	9.75± 0.77	1.18± 0.24	2.33± 0.05	82.51 ± 0.04	4.22± 0.92	ND	ND
	Hass	W	ND	ND	8.43± 0.16	1.14± 0.03	2.82± 0.81	82.77 ± 0.33	4.84± 0.35	ND	ND
		M	ND	ND	7.87± 0.5	0.77± 0.04	2.96± 1.07	83.6± 0.15	4.72± 0.79	ND	0.08± 0.12
Mid	Car	W	ND	ND	8.1± 0.23	1.04± 0.11	2.17± 0.06	83.69 ± 0.22	4.84± 0.57	ND	0.17± 0.01
		M	ND	ND	7.84± 0.57	0.71± 0.22	2.48± 0.44	84.23 ± 0.17	4.63± 0.76	ND	0.11± 0.05
	Hass	W	ND	ND	7.92± 0.48	1.02± 0.08	2.36± 0.2	83.28 ± 0.61	5.25± 0.27	ND	0.17± 0.01
		M	ND	ND	7.64± 0.48	0.72± 0.05	2.06± 0.06	84.31 ± 0.69	5.1± 0.12	ND	0.16± 0.03
Late	Car	W	ND	ND	7.85± 0.7	0.94± 0.22	2.14± 0.14	83.37 ± 0.37	5.71± 1.26	ND	ND
		M	ND	ND	8.13± 0.44	0.64± 0.12	2.08± 0.54	84.42 ± 0.82	4.73± 0.78	ND	ND
	Hass	W	ND	ND	8.57± 1.41	0.97± 0.17	2.7± 1.13	81.71 ± 1.39	6.05± 0.32	ND	ND
		M	ND	ND	7.58± 0.2	0.79± 0.26	2.29± 1.08	84.01 ± 1.46	5.3± 0.87	ND	0.02± 0.04

Table S4.8. Sterols dataset for experimental design two, Irvine location. Samples reported as %±SD. Each sterol is abbreviated; all end with -sterol except for delta-7-stigmastenol. The first column is harvest time, followed by cultivar (Car = Carmen) and processing, where M is mesocarp and W is whole. Samples in red did not meet proposed standards.

			Chole	Bras-sica	Campe	Stigma	Clero	B-sito	Δ-5-avena	Δ-7-stigma	Δ-7-avena
Very early	Car	W	ND	ND	9.01± 0.91	1.28± 0.19	2.86± 0.88	82.53± 0.68	4.33±1.3	ND	ND
		M	ND	ND	9.02± 0.19	0.78± 0.04	1.78± 0.21	84.53± 0.73	3.8±0.16	0.09± 0.13	ND
	Hass	W	ND	ND	9.49± 0.2	1.11± 0.07	2.18± 0.38	82.88± 0.98	4.34±0.34	ND	ND
		M	ND	ND	9.55± 0.3	0.65± 0.07	2.21± 0.1	83.21± 0.55	4.37±0.09	ND	ND
Early	Car	W	ND	ND	7.71± 1.35	0.76± 0.57	1.6± 0.59	83.66± 0.95	6.2±1.66	ND	0.06± 0.09
		M	ND	ND	7.67± 0.1	0.79± 0.08	1.65± 0.4	84.03± 1.57	5.86±2.15	ND	0±0
	Hass	W	ND	ND	7.9± 0.25	0.9± 0.14	2.13± 0.18	82.15± 0.29	6.7±0.33	ND	0.21± 0.06
		M	ND	ND	7.63± 0.39	0.61± 0.11	2.1± 0.1	82.34± 0.45	7.18±0.67	ND	0.13± 0.13
Early/ mid	Car	W	ND	ND	7.79± 0.4	1.03± 0.16	1.92± 0.13	83.89± 0.58	5.31±1.17	ND	0.06± 0.09
		M	ND	ND	7.46± 0.38	0.63± 0.14	1.92± 0.09	84.36± 0.42	5.64±0.19	ND	ND
	Hass	W	ND	ND	7.08± 0.19	0.68± 0.23	1.9± 0.06	83.73± 0.35	6.61±0.65	ND	ND
		M	ND	ND	6.98± 0.36	0.53± 0.14	2.42± 1.01	83.55± 1.04	6.52±0.4	ND	ND
Mid	Car	W	ND	ND	6.8± 0.37	0.93± 0.17	2±0.02	84.45± 0.14	5.7±0.43	ND	0.12± 0.01
		M	ND	ND	6.18± 0.24	0.66± 0.13	1.97± 0.03	84.79± 0.47	6.4±0.82	ND	ND
	Hass	W	ND	ND	6.54± 0.23	0.84± 0.02	2.06± 0.92	84.27± 0.36	6.26±0.46	ND	0.03± 0.05
		M	ND	ND	6.2± 0.09	0.55±0 .06	1.85± 0.15	84.47± 0.5	6.89±0.53	ND	0.04± 0.06
Mid/ late	Car	W	ND	ND	6.09± 0.47	0.81± 0.41	2.02± 0.08	84.37± 0.63	6.46±1.37	ND	0.24± 0.06
		M	ND	ND	6.02± 0.07	0.68± 0.15	2±0.15	84.94± 0.39	6.14±0.65	ND	0.22± 0.03
	Hass	W	ND	ND	5.96± 0.38	0.78± 0.1	2.09± 0.18	83.89± 0.61	7.05±0.11	ND	0.23± 0.05
		M	ND	ND	6.07± 0.18	0.68± 0.17	2±0.18	84.59± 0.24	6.47±0.7	ND	0.2± 0.02
Late	Car	W	ND	ND	5.94± 0.28	0.78± 0.05	2.23± 0.02	85.87± 0.76	4.98±0.98	ND	0.21± 0.03
		M	ND	ND	6.04± 0.39	0.83± 0.42	2.12± 0.05	85.3± 0.85	5.46±0.03	ND	0.25± 0.03
	Hass	W	ND	ND	6.11± 0.06	0.83± 0.07	2.05± 0.02	85.64± 0.23	5.14±0.22	ND	0.23±0
		M	ND	ND	6.14± 0.04	0.64± 0.08	2.14± 0.06	84.94± 0.13	5.86±0.08	ND	0.27± 0.04

Chapter 5

Cis-vaccenic acid: new maker to detect seed-oil adulteration in avocado oil

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Abstract

Efforts to ensure avocado oil purity have been made and standards are currently being developed, however, challenges still exist with differentiating avocado oil from high oleic seed oils, such as sunflower and safflower oils. Cis-vaccenic acid or C18:1 (n-7) is an oleic fatty acid isomer that has significantly different concentrations in avocado oil compared to high oleic sunflower and safflower oils, as well as other adulterants. High oleic safflower oil has cis-vaccenic values <0.75 % of total fatty acid content and high oleic sunflower <1.3 % total fatty acid content, while avocado oil has concentrations ranging from 5.0 % to 7.5 %. Standards on fats and oil typically only require for total oleic acid to be reported (C18:1 (n-9) plus C18:1 (n-7)), however, this work demonstrates that there is an immediate need to consider including a separate standard for cis-vaccenic content in avocado oil to aid in detecting adulteration, particularly with seed oils.

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Background

Avocado oil is a high value edible oil that is growing rapidly in popularity. With its growth it will be important to ensure pure oil can be differentiated from economically motivated adulterations (Green and Wang 2020). The most common methods currently used to determine purity in oils are fatty acid profile and sterols profile, with fatty acid profile being more widely used and well researched (Yanty et al. 2011; Ozdemir and Topuz, 2004; Jorge et al. 2015; Manaf et al. 2019; Fernandes et al. 2018; Tan et al. 2017, Slater et al. 1975). However, it can still be difficult to detect adulteration of seed oils; namely high oleic sunflower and high oleic safflower oils in avocado oil (Green and Wang, 2020). It is necessary to work on developing different methods or indicators to differentiate avocado oil from these common adulterants. Cis-vaccenic acid, C18:1 (n-7), is an isomer of the more common oleic fatty acid (C18:1 (n-9)). Oftentimes these compounds coelute and are thus quantified together as the C18:1 total (Ratovehery et al. 1988).

It has been shown that cis-vaccenic acid is synthesized via the elongation of palmitoleic fatty acid (Shibahara, 1989). Shibahara (1990) also demonstrated cis-vaccenic can be formed via enzymatic double-bond shifting between C18:1 (n-9) and (n-7). However, since this initial work on cis-vaccenic acid, little has been done to further examine this compound. Several studies have quantified C18:1 (n-7) and (n-9) and their ratios in fruits and seeds (Sebedio and Ackman, 1979; Mukherjee and Kiewitt, 1980; Destaillats et al. 2002), with even fewer studies, to our knowledge, that worked to understand cis-vaccenic acid separately from oleic acid in avocados (Plaza et al. 2009; Zuazo et al. 2021; Wang et al. 2020).

Fatty acid profile is used to determine the purity of edible oils and is included in the proposed avocado oil standard by CODEX Alimentarius, an international food standards

agency (CODEX, 2021). In these standards, oleic fatty acid is reported as the sum of C18:1 (n-9) and (n-7). Because cis-vaccenic acid can be determined using an already existing method, it is feasible for this fatty acid to be quickly and easily included in standards for avocado oil.

Materials and Methods

Experimental design

The authentic avocado samples used in this study (n=68) were previously described in Green and Wang, 2022. In brief, the avocado oils were made either by cold-pressed using a lab-scale mechanical extractor or solvent extracted with hexane. A variety of factors were accounted for in these avocado oils including region harvested (California and Mexico), harvest time, grade of fruit used, cultivar, and using whole fruit or flesh to make the oil. The other oils used in this study (high oleic sunflower, high oleic safflower, soybean, and canola oil) are potential adulterants that can be present in avocado oil. Ten samples were gathered for each of these "adulterant" oils and were confirmed to be pure (for example, that every canola oil sample was 100 % canola oil) and then were used for this study.

Fatty acid profile

The IOC official method for the determination of the fatty acid methyl esters by gas chromatography (COI/T.20/ Doc. No 33/Rev.1, 2017) was used for fatty acid profile analysis with some modifications. In brief, approximately 20 μL of oil was mixed with 3 mL of hexane and mixed, followed by the addition of 200 μL of 2M methanolic KOH. After vigorously mixing for 1 min samples were left until the top solution was clear. The organic layer was filtered before GC analysis using 0.2-micron PTFE filters. The GC-FID analysis was conducted on an Agilent 7890A GC (Agilent Technologies). A 90 m × 250 μm × 0.25 μm DB-FastFAME capillary

column (Agilent Technologies) was used to achieve the separation of individual fatty acids and isomers, specifically C18:1 (n-7) and (n-9). The injection volume was 1.0 μL and helium was used as a carrier gas at a flow rate of 1.9 mL min^{-1}. The injector temperature was held at 260 °C at a split ratio of 30. The GC oven program was initially held at 35°C for 1 min; then ramped at 65°C min^{-1} to 200 °C and held for 14 min, followed by a ramp of at 2.5°C min^{-1} to 210 °C, which was held for 5 min. The last ramp was at 12°C min^{-1} to 230°C and held for 20 min, giving a total run time of 49.2 min. The FID temperature was 260°C. The detector gas consisted of hydrogen (flow rate: 40 mL min^{-1}), air (flow rate: 400 mL min^{-1}), and helium make up gas (flow rate: 25 mL min^{-1}). Peak identification was performed using a 37-component FAME reference standard mix and a cis-vaccenic acid analytical standard (MilliporeSigma).

Statistical analysis

Statistical analysis was performed using ANOVA in R, with significance being determined by an alpha level of 0.05 using the Tukey test.

Results and Discussion

Cis-vaccenic acid content in avocado oil and seed oils

Figure 5.1 shows the concentration of C18:1 (n-7) (cis-vaccenic acid) as percent of total fatty acid content in pure avocado oil compared to four common adulterant oils (canola, soybean, high oleic safflower, high oleic sunflower) found in avocado oil. Sebedio and Ackman, 1979 determined the amount of C18:1 (n-7) and (n-9) in canola oil and reported comparable values to the ones seen in this study. All the adulterant oils have under 3.5 % cis-vaccenic acid, with avocado oil ranging from 5-7.5 %, averaging at 6.45 %, similar to the ~6 % average seen in Wang et al. 2020. Avocado oil had significantly different cis-vaccenic acid content when compared to each of the other oil types, p-value <0.0001 in all cases according to the Tukey test.

Cis-vaccenic acid has more staggering differences between avocado oil and the seed oils in this work, with high oleic safflower oil under 1.0 % and high oleic sunflower oil under 1.3 %. Perona et al. 2005 also determined the amount of cis-vaccenic acid in high oleic sunflower oil, via a study on rat diets, and found similarly low values. Currently in standards, the C18:1 (oleic acid) content is reported as the sum of C18:1 (n-9) and C18:1 (n-7), thus the ratio of C18:1 (n-9/n:7) was also investigated (Figure 5.2a). Avocado oil contains the lowest ratio (5.7-13) of all the oils, with high oleic sunflower and safflower oils averaging around 100. This ratio was significantly different, p-value <0.0001, between avocado oil and each of the other potential adulterant oils according to the Tukey test. Figure 5.2b shows log-transformed data of Figure 5.2a to better see the variance in the data. There are more outliers (marked by black circles) for the avocado oils likely because the sample size was larger for avocado oil than the other oil types.

Typical values seen in authentic avocado oil

If cis-vaccenic acid were to be included in standards either as the amount of the indicator itself, or as the ratio of C18:1 (n-9/n-7) ranges would need to be established. Based on the 68 authentic samples in this study, there were only two samples below 5.5 % cis-vaccenic acid and were likely outliers, thus the proposed standard range could be from 5.5-7.5 %. It will be necessary to ensure the lower end of this range is carefully adjusted; if it goes too low, it risks overlapping with other oils and not being able to detect adulteration as well. If the ratio of C18:1 (n-9/n-7) is used the proposed range based on this study would be 5.7-11.5. The same two samples that had low cis-vaccenic acid values (around 5%) also had higher ratios (12-13), which were considered outliers and thus excluded. It should be noted that 94% of avocado oil samples had ratios equal to or less than 10.

Like any standard, continuing research is needed to ensure ranges are accommodate varying regions and climates. Zuazo et al. 2021 found that cis-vaccenic content acid in avocados can decrease with water stress, an important finding that should continue to be monitored. Our work developed ranges based on lab-made oils from California and Mexico fruits. We also tested five pure avocado oils sent in from producers from New Zealand (two samples), Kenya (one sample), and South Africa (two samples), to see if these oils also fit into the same ranges as seen in our samples. All five of these oils had cis-vaccenic acid values between 5.5-7.5 % and had a ratio of C18:1 n-9/n-7 between 5.7-11.5. These results are promising in the potential of cis-vaccenic acid to be used as an adulteration marker. The proposed range of cis-vaccenic acid values developed from the samples in this study also accommodates other avocado-oil producing regions.

Creating blended samples

There are already known markers that can distinguish avocado oil from canola and soybean oils (namely brassicasterol content and linoleic content, respectively), which can be used to detect adulterations with these oils. However, it is more difficult to detect the adulteration of avocado oil with high oleic safflower and sunflower oils. To see how well cis-vaccenic acid could be used as a marker to determine the mixing of avocado with seed oils, blends were made with a representative sample of avocado oil from this study with either a high oleic sunflower or high oleic safflower oil. Figure 5.3 shows the results of these blends where the cis-vaccenic acid content decreases from 6.74 – 0.79 % and 6.74 – 0.69 % for pure avocado oil to high oleic sunflower oil, and safflower oil, respectively. The black dash lines across the plot are our suggestions for the proposed ranges of cis-vaccenic acid for pure avocado oil. When using this as a minimum, adulterations of 25 % with high oleic sunflower or safflower oil can be

detected, if not slightly lower, as the 5.5 % minimum line lies at around 18 % each adulterant (82 % avocado oil). Figure 5.4 shows how the ratio of C18:1 n-9/n-7 varies with the set of blends. Figure 5.4b zooms in on the blends with greater than 50 % avocado oil so the lower levels of adulteration can be seen more easily. The black dash lines indicate the proposed range of ratios for pure avocado oil, which can detect adulteration at around 25 % (75 % avocado oil) or greater for both high oleic safflower and sunflower oils. Based on these figures, the amount of cis-vaccenic acid can detect adulteration at slightly lower amounts compared to using the C18:1 (n-9/n-7) ratio.

Table 5.1 shows the fatty acid profile for blends of avocado oil with these seed oils at four different concentrations, to further illustrate the utility of cis-vaccenic acid. For both high oleic sunflower and safflower oils, our proposed range of cis-vaccenic acid values can differentiate blends including 25 % of the adulterant which the current proposed fatty acid standards cannot. Furthermore, in the case of high oleic safflower oil a blend including 50 % of this adulterant could go undetected with fatty acid standards except for its low, 3.4 % cis-vaccenic acid content.

Conclusions

The results from this work demonstrate that avocado oil has uniquely high amounts of cis-vaccenic acid (5.5-7.5 %) and is the first study to show that cis-vaccenic acid could serve as an important maker of seed oil adulteration in avocado oil. It is especially useful for detecting high oleic seed oils which traditionally has been difficult to detect the adulteration of avocado oil, without the need to conduct a different set of analytical method or the requirement for more instrumentations. In terms of standard inclusion and limit adoption, this study demonstrated that using the amount of cis-vaccenic acid compared to the ratio of C18:1

(n-9/n-7) was slightly better at detecting adulteration. In addition, several products from other avocado oil producing countries were tested, which also fit into the ranges seen from the California and Mexico avocado oils used in this study. Future work could aim to gain understanding how natural variances such as cultivars, maturation, post-harvest, and processing affect cis-vaccenic acid and how it (particularly in relation to other fatty acids) can change in avocado oil, in addition to including larger sample sizes of other avocado oil producing regions. This would help further adjust the range of allowable standard for cis-vaccenic acid so it could become a more sensitive marker (i.e. detecting high oleic seed oils at less than 10%) and accommodate the effects of natural variables.

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Competing Interests

The authors have no competing interests to declare.

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Tables

Table 5.1. Fatty acid profile for blends of a pure avocado oil with high oleic sunflower or safflower oil. Values in blue meet the current proposed CODEX standard for pure avocado oil, despite the presence of high oleic sunflower or safflower oil. Values in red do not meet the current proposed CODEX standard.

Fatty acid	Proposed CODEX limits (%)	% High oleic sunflower oil				% High oleic safflower oil			
		10	25	50	75	10	25	50	75
C14:0	ND-0.3	ND	ND	ND	ND	ND	ND	0.1	0.1
C16:0	11.0-26.0	16.2	14.1	11.0	7.6	16.3	14.3	11.4	8.3
C16:1	4.0-17.1	7.4	6.1	4.2	2.1	7.4	6.2	4.0	2.1
C18:0	0.1-1.3	0.7	1.1	1.6	2.2	0.6	0.9	1.3	1.7
C18:1	42.0-75.0	63.7	67.3	72.8	79.0	62.9	65.6	69.8	74.0
C18:2	7.8-19.0	10.8	10.0	8.8	7.5	11.5	11.7	12.1	12.5
C18:3	0.5-2.1	0.9	0.7	0.5	0.3	0.9	0.8	0.6	0.4
C20:0	ND-0.7	ND	0.2	0.2	0.2	0.1	0.2	0.3	0.4
C20:1	ND-0.3	0.2	0.2	0.3	0.3	0.2	0.3	0.3	0.3
C22:0	ND-0.5	0.1	0.3	0.5	0.8	0.0	0.1	0.2	0.2
Our proposed limits for C18:1 (n-7 and n-9/n-7)									
C18:1 n-7	5.5-7.5	5.9	5.0	3.6	2.1	5.9	5.1	3.4	1.9
C18:1 (n-9/n-7)	5.7-11.5	9.9	12.4	19.0	37.3	9.7	12.0	19.5	37.0

Figures

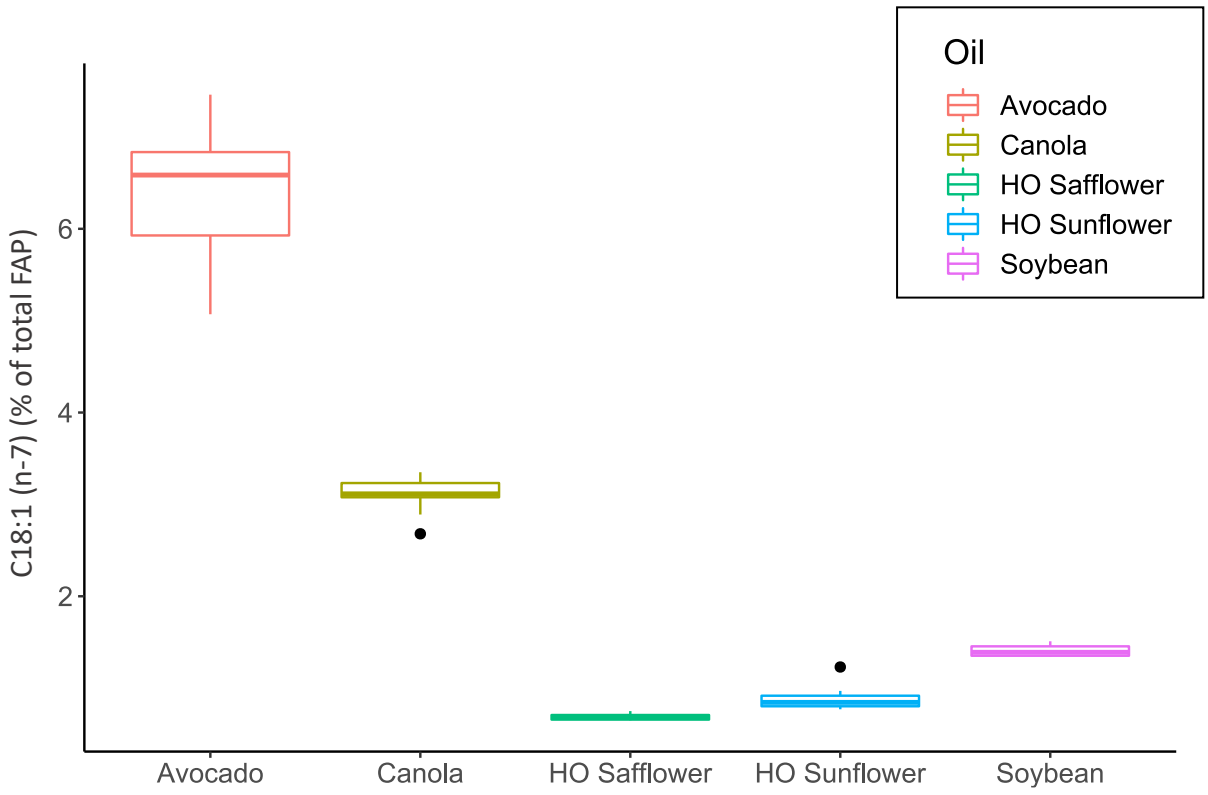


Figure 5.1. Box and whisker plot showing the percent cis-vaccenic acid (C18:1 n-7) in avocado oil compared to other potential adulterant oils. HO = high oleic.

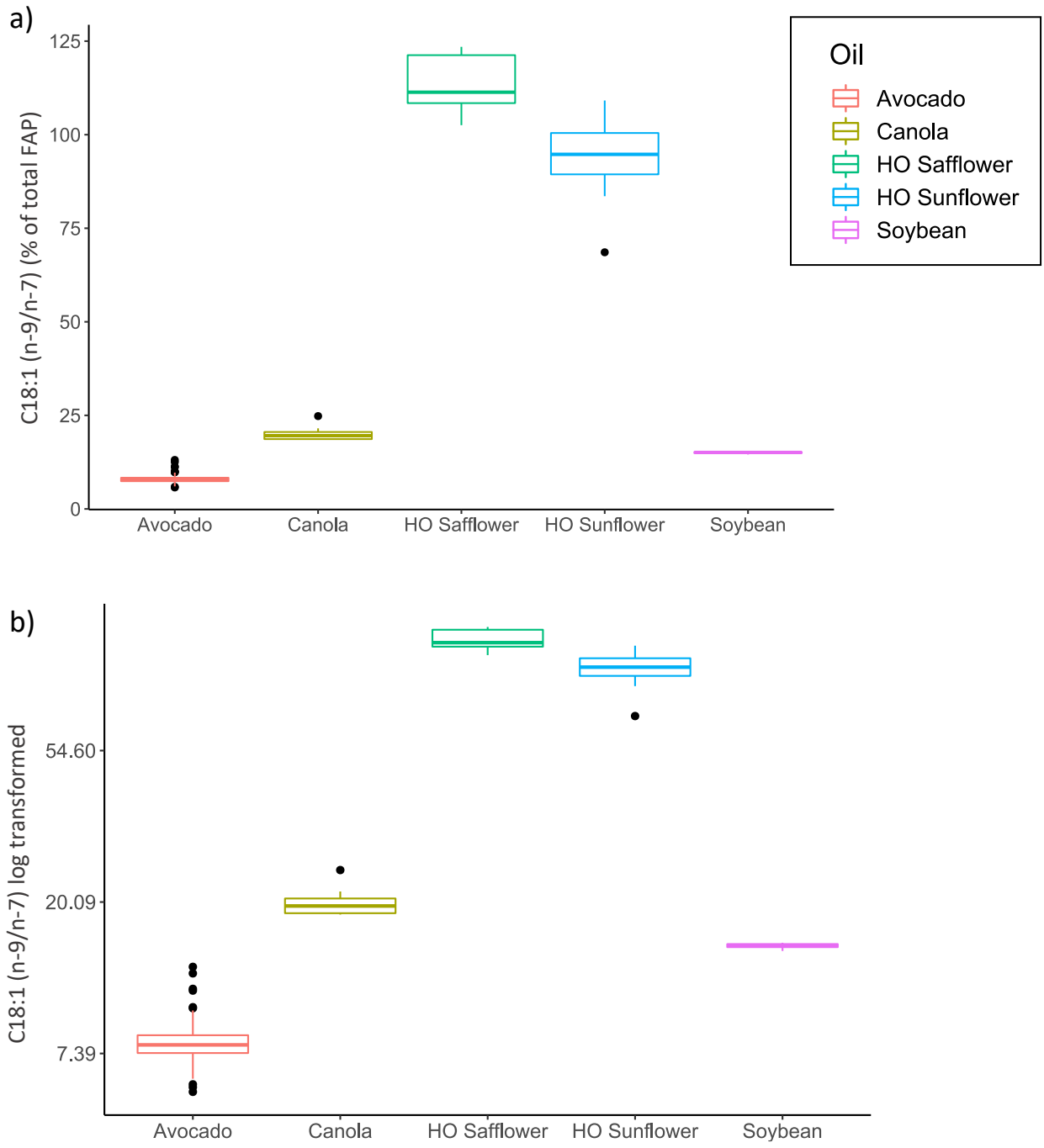


Figure 5.2. a) Box and whisker plot showing the ratio of C18:1 n-9: n-7 in avocado oil compared to other potential adulterant oils where black dots indicate outliers in each dataset. HO = high oleic. b) Box and whisker plot of the same data seen in (a) but log transformed to better see the variance within each oil group.

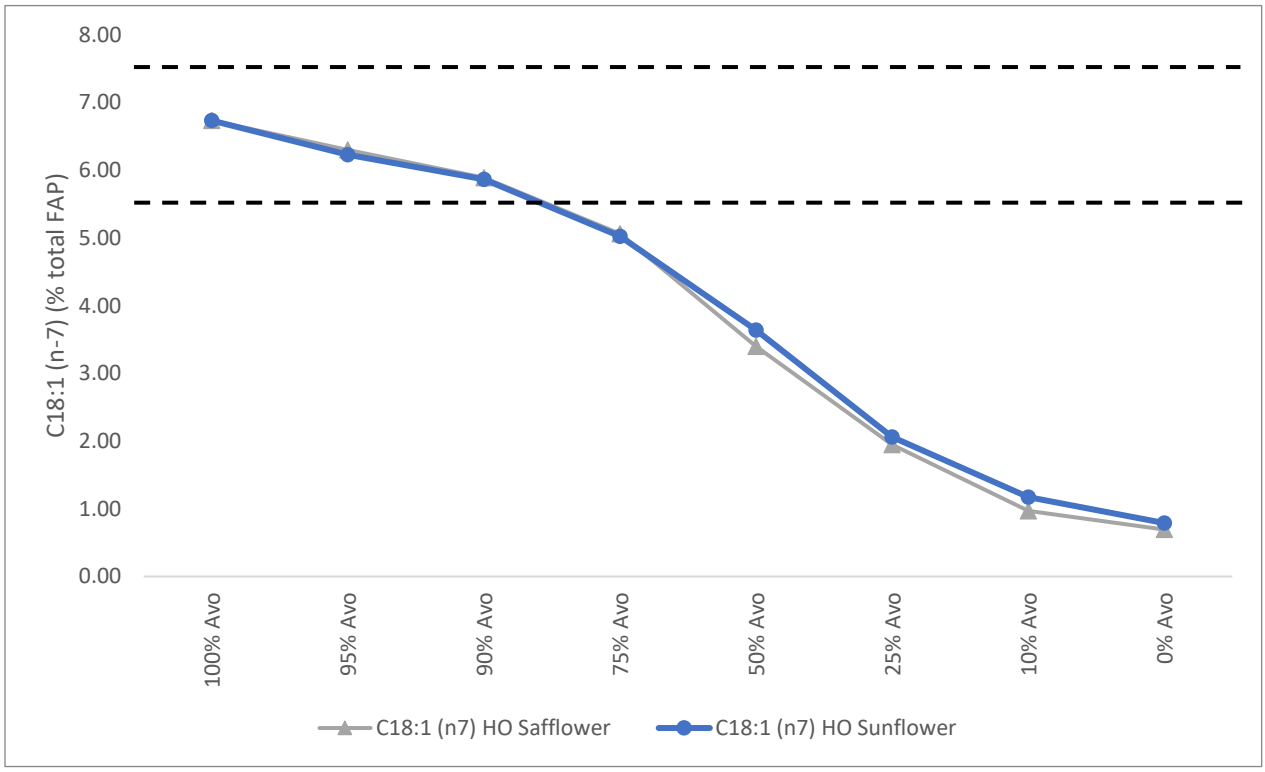
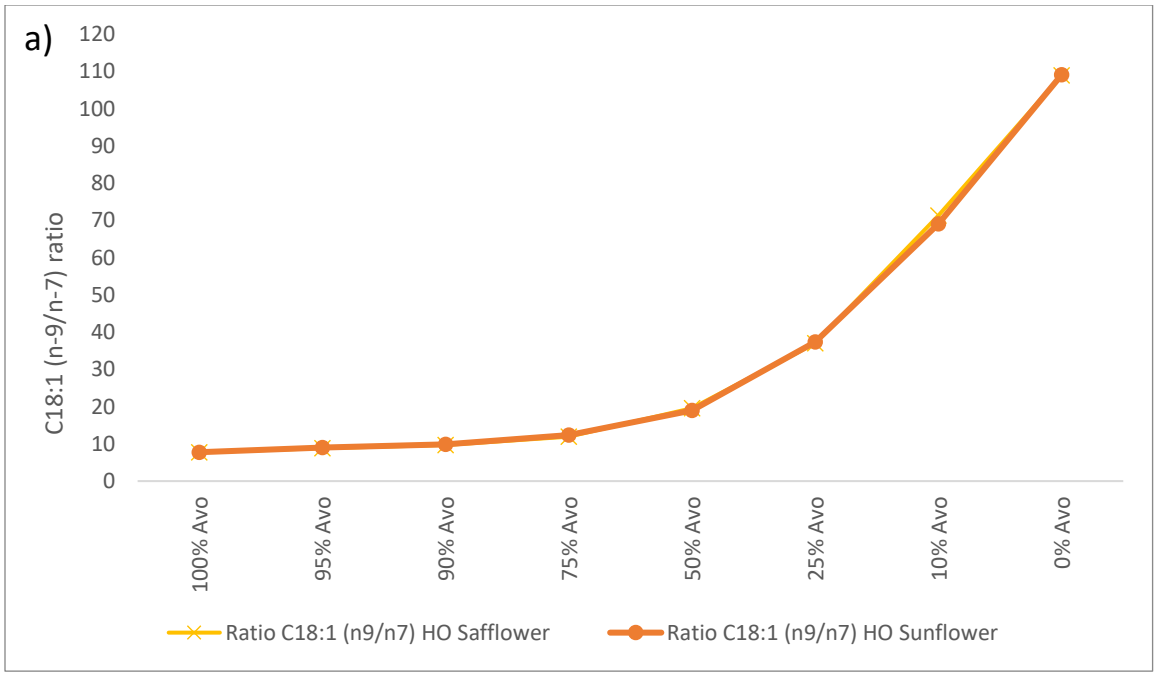


Figure 5.3. Plot demonstrating the change in cis-vaccenic acid content in an avocado oil adulterated at varying percentages with either high oleic sunflower or high oleic safflower oil. Black lines indicate the typical range of cis-vaccenic acid seen in pure avocado oils. Avo = Avocado oil, HO = High oleic.



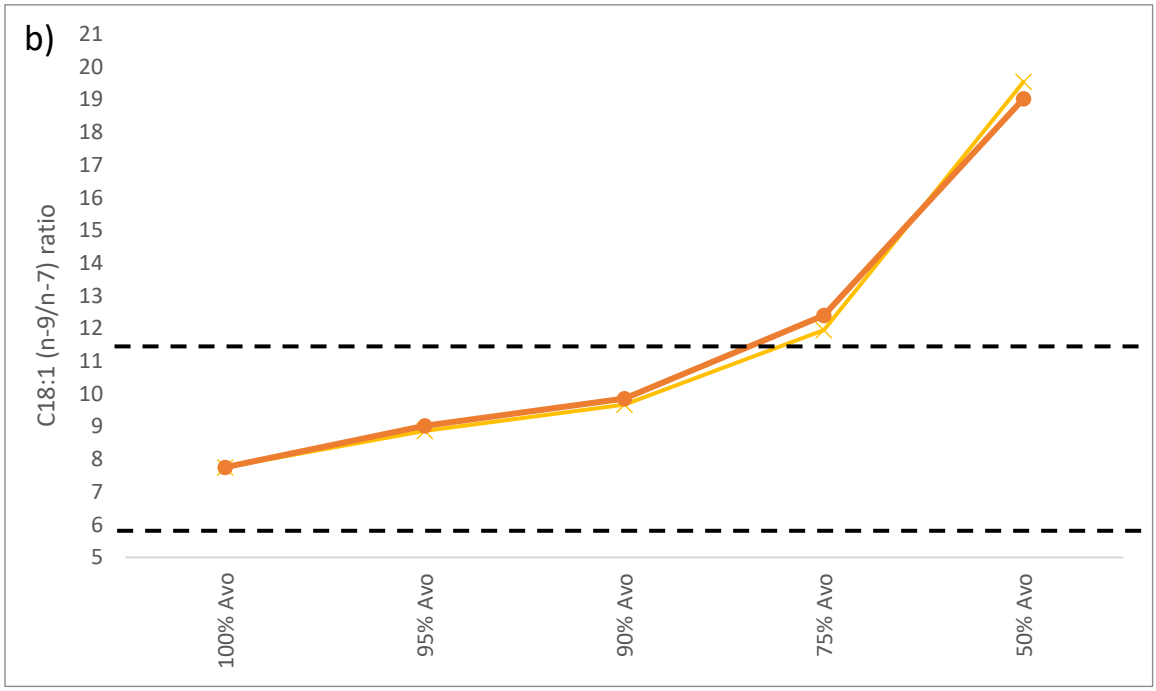


Figure 5.4. a) Plot demonstrating the change in the ratio of C18:1 n-9: n-7 in an avocado oil adulterated with varying percentages with either high oleic sunflower or high oleic safflower oil. b) Black lines indicate the typical range of n-9: n-7 seen in pure avocado oils. Avo = Avocado oil, HO = High oleic

Supplementary Tables

Table S5.1. C18:1 (n-7) and (n-9) values for avocado oils used in this study presented as average (%) ± SD, which were originally discussed in experimental design one in Green and Wang 2022. This set includes 32 of the 68 avocado oils used in this study.

Region	Harvest Time	Grade	Processing	C18:1 (n9)	C18:1 (n7)	C18:1 n-9/n-7
CA	Early	G1	Whole	56.12±0.02	6.79±0	8.26±0
			Mesocarp	55.78±0.01	6.83±0	8.17±0
		G4	Whole	53.48±0.01	6.67±0	8.01±0
			Mesocarp	53.97±0.01	6.59±0	8.19±0
	Mid	G1	Whole	52.78±0.08	6.87±0.01	7.68±0
			Mesocarp	53.23±0.03	6.85±0.01	7.77±0
		G4	Whole	48.99±0.02	7.13±0	6.87±0.01
			Mesocarp	55.57±0.01	6.91±0	8.05±0
	Late	G1	Whole	63.73±2.73	6.64±0.29	9.6±0
			Mesocarp	61.8±0.04	6.3±0	9.82±0
		G4	Whole	51.97±0.79	6.81±0.11	7.63±0.24
			Mesocarp	55.5±0.01	6.46±0.01	8.59±0.01
MX	Early	G1	Whole	46.29±0	6.21±0.01	7.45±0.01
			Mesocarp	47.05±0.01	6.56±0	7.17±0
		G4	Whole	48.22±0.01	5.58±0	8.64±0
			Mesocarp	46.17±0.02	5.95±0.01	7.76±0
	Early/mid	G1	Whole	55.91±0.03	5.61±0	9.96±0.01
			Mesocarp	54.7±0.07	5.64±0	9.7±0.02
		G4	Whole	52.66±0.85	6.87±0.11	7.67±0
			Mesocarp	52.21±0.01	6.64±0.01	7.86±0.01
	Mid	G1	Whole	66.34±0.01	5.07±0	13.09±0.01
			Mesocarp	65.76±0.02	5.24±0	12.54±0.01
		G4	Whole	61.51±0.01	5.5±0	11.19±0.01
			Mesocarp	62.2±0.01	5.49±0	11.32±0.01
	Mid/late	G1	Whole	52.24±1.95	6.74±0.16	7.76±0.1
			Mesocarp	57.9±0	7.2±0	8.04±0
		G4	Whole	55.58±0.04	5.66±0.01	9.81±0
			Mesocarp	56.99±0.01	5.68±0.01	10.03±0.02
	Late	G1	Whole	51.04±0.23	6.55±0.04	7.8±0.01
			Mesocarp	51.86±0.05	6.61±0.01	7.85±0
		G4	Whole	52.09±0	6.79±0	7.67±0
			Mesocarp	53.49±0	6.7±0	7.98±0

Table S5.2. C18:1 (n-7) and (n-9) values for avocado oils used in this study presented as average (%) ± SD, which were originally discussed in experimental design two in Green and Wang 2022. This set includes 36 of the 68 avocado oils used in this study.

Region	Harvest time	Cultivar	Processing	C18:1 (n9)	C18:1 (n7)	C18:1 (n-9/n-7)
Lindcove	Early	Carmen	Whole	43.33±0.44	6.41±0.24	6.76±1.81
			Mesocarp	43.86±0.47	6.62±0.26	6.63±1.82
		Hass	Whole	44.92±0.06	5.83±0	7.7±25.45
			Mesocarp	43.35±0.09	5.86±0.01	7.4±6.98
	Mid	Carmen	Whole	49.01±0.63	5.91±0.1	8.3±6.47
			Mesocarp	50.06±1.25	5.92±0.27	8.46±4.68
		Hass	Whole	48.91±2	5.93±0.35	8.24±5.76
			Mesocarp	48.69±1.23	6.0±0.37	8.12±3.31
	Late	Carmen	Whole	51.53±1.11	5.76±0.09	8.95±12.72
			Mesocarp	52.96±0.87	5.68±0.04	9.32±23.08
		Hass	Whole	51.02±2.47	5.75±0.38	8.88±6.52
			Mesocarp	51.27±2.6	5.86±0.44	8.75±5.98
Irvine	Very Early	Carmen	Whole	47.77±0.11	6.19±0.05	7.72±2.5
			Mesocarp	47.39±0.24	6.38±0.05	7.42±5.08
		Hass	Whole	46.19±0.05	6.1±0.03	7.57±1.83
			Mesocarp	46.16±0.26	6.23±0.05	7.41±4.79
	Early	Carmen	Whole	49.9±2.59	6.11±0.2	8.17±12.96
			Mesocarp	50.3±1.62	6.47±0.13	7.78±12.46
		Hass	Whole	47.83±1.04	7.01±0.21	6.82±4.9
			Mesocarp	48.82±1.09	6.77±0.24	7.21±4.45
	Early/mid	Carmen	Whole	52.39±0.76	6.58±0.11	7.96±6.66
			Mesocarp	52.22±2.52	6.51±0.04	8.02±60.58
		Hass	Whole	50.85±1.32	6.97±0.01	7.3±123.03
			Mesocarp	50.86±2.7	7.02±0.27	7.24±10.17
	Mid	Carmen	Whole	53.84±1.13	6.61±0.02	8.14±75.46
			Mesocarp	52.71±1.61	6.83±0.05	7.72±30.11
		Hass	Whole	52.63±0.98	7.11±0.09	7.4±11.35
			Mesocarp	51.58±1.02	7.44±0.44	6.93±2.34
	Mid/late	Carmen	Whole	53.84±1.13	6.61±0.02	8.14±75.46
			Mesocarp	52.71±1.61	6.83±0.05	7.72±30.11
		Hass	Whole	48.33±1.16	7.4±0.08	6.53±15.06
			Mesocarp	47.61±0.85	7.36±0.24	6.47±3.5
	Late	Carmen	Whole	44.49±0.82	7.11±0.03	6.25±24.52
			Mesocarp	43.87±0.36	7.28±0.09	6.03±3.82
		Hass	Whole	43.64±2.56	7.38±0.38	5.92±6.73
			Mesocarp	42.8±1.01	7.46±0.22	5.74±4.67

Table S5.3. C18:1 (n-7) and (n-9) values for oils that are commonly used as adulterants in avocado oil, presented as average (%) ± SD.

	C18:1 (n9)	C18:1 (n7)	C18:1 (n-9/n-7)
Soybean	22.54±0.01	1.49±0	15.17±0
	22.1±0	1.44±0	15.35±0
	20.95±0	1.38±0	15.18±0.01
	20.03±0.02	1.34±0	14.91±0.02
	20.85±0.02	1.4±0	14.94±0.01
	20.28±0.01	1.35±0.01	15.01±0.1
	20.71±0	1.36±0	15.27±0.01
	20.59±0.02	1.35±0	15.23±0
	21.19±0.02	1.46±0	14.54±0.02
	21.01±1.71	1.42±0.12	14.79±0.03
Safflower	77.71±0.05	0.69±0	112.18±0.59
	75.56±0.08	0.69±0	108.98±0.63
	77.84±0.08	0.7±0.01	110.54±2.36
	82.33±0.42	0.68±0.01	121.9±1.91
	76.8±0.01	0.72±0	107.2±0.46
	79.67±0.02	0.65±0	123.48±0.15
	77.19±0.03	0.75±0	102.54±0.21
	77.43±0.03	0.65±0	119.34±0.31
	76.5±0.07	0.71±0	108.25±0.1
	79.45±0.07	0.65±0	122.18±0.53
Sunflower	80.98±0.07	0.97±0	83.57±0.09
	82.67±0.01	0.82±0.01	101.34±0.86
	84.24±0.11	1.23±0	68.58±0.15
	77.63±0.05	0.8±0	97.18±0.21
	83.59±0.02	0.77±0	108.28±0.28
	80.76±0.06	0.91±0	88.95±0.1
	86.17±0.01	0.79±0.01	109.14±0.81
	79.66±0.44	0.81±0.01	97.78±0.28
	83.49±0.01	0.92±0.01	90.77±0.82
	80±0.01	0.87±0	92.32±0.18
Canola	61.91±0.04	3.12±0	19.85±0.01
	59.68±0.04	3.21±0.02	18.58±0.1
	59.82±0	3.1±0	19.29±0
	60.12±0.02	3.24±0	18.54±0.01
	60.43±0.02	3.27±0.02	18.49±0.1
	63.72±0.03	3.07±0	20.74±0.01
	62.37±0.01	3.11±0	20.03±0
	66.4±0.41	2.68±0	24.81±0.11
	63.32±0.09	3.35±0.01	18.89±0.01
	62.28±0.03	2.89±0	21.53±0.01

Table S5.4. C18:1 (n-7) and (n-9) values for pure avocado oils acquired from producers in other common avocado-oil producing regions around the world, presented as average (%) ± SD.

	C18:1 (n9)	**C18:1 (n7)**	**C18:1 (n-9/n-7)**
Kenya_1	52.18±0	5.55±0	9.41±0
New Zealand_1	62.76±0.01	5.98±0	10.49±0.01
New Zealand_2	58.59±0	6.43±0	9.12±0
South Africa_1	57.44±0.01	6.09±0	9.44±0
South Africa_1	52.72±0.03	6.2±0	8.5±0.01

Chapter 6

A rapid method for the detection of extra virgin olive oil adulteration using UHPLC-CAD profiling of triacylglycerols and PCA

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Abstract

Adulteration in extra virgin olive oil (EVOO) is a common fraud due to its superior value over other edible oils. Traditional methods of fatty acid and sterol profiling for detecting adulteration demand large amounts of time and excessive use of labor and solvents therefore, new methodologies are needed to determine the authenticity of EVOO that are both time-efficient and cost-effective. Ultra-high-performance liquid chromatography (UHPLC) with charged aerosol detection (CAD) was employed to characterize EVOO along with potential adulterant oils based on their triacylglycerol (TAG) profiles. Statistical analysis of these TAGs using principal component analysis (PCA) allows for a rapid approach to determine EVOO authenticity. Using

this approach, adulteration of EVOO with cheaper vegetable and seed oils and lower-quality olive oils had detection limits at or below 10%, depending on the adulterant. Compared to traditional methods, UHPLC-CAD with PCA involves minimal sample preparation combined with fast analysis, for a rapid determination of EVOO authenticity.

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Background

Extra virgin olive oil (EVOO) is the highest grade of olive oil and is in higher demand due to its desirable taste and nutritional benefits compared to other edible oils. Unfortunately, due to its superior economic value, adulteration by addition of cheaper vegetable oils (soybean, canola, sunflower, safflower, etc.) or lower quality olive oils (ordinary olive oil, refined olive oil, etc.) persists and is often a challenge to be detected and contained immediately, especially for imported olive oil at customs or with trading companies (Bayramer et al., 2018; Camin et al., 2016; Esteki et al., 2019). Adulterated olive oil labeled and sold as EVOO not only causes economic detriment to genuine olive oil producers and traders in the industry, but also creates trade barriers between countries and increases confusions among consumers. Thus, it is crucial to ensure that EVOO on the market is authentic not only to maintain its high economic value, but also to make certain that consumers receive the safe and high-quality products that they have paid for. Furthermore, some adulterants can be detrimental to human health, thus it is imperative that consumers are not exposed to such potential toxins (Arlorio et al., 2010).

Currently, measurement of fatty acid and sterol profiles are the most commonly used official methods, established by the International Olive Council (IOC), for analyzing olive oil purity (IOC, 2017a; IOC, 2017b). However, these methods involve time-consuming extractions, excessive solvent use, and lengthy data analysis (IOC, 2017a, 2017b). In addition, the measurement of fatty acids and sterols may not always reflect the correct categorization of olive oil in order to adequately determine if an adulterant is present (Conte et al., 2019). The time and resources necessary for these analyses often mean they cannot be easily completed by customs on-site or companies in-house, and samples must be sent to accredited testing laboratories to be analyzed. With the stable increase of global olive oil imports, especially in countries where olive

oil is a relatively new product to consumers with less experience and knowledge (IOC, 2019), there is an urgent demand for a time-efficient and cost-effective adulteration detection solution to facilitate with or replace the traditional olive oil purity analyses.

Triacylglycerols (TAGs) are the principal component which make up 90% of the compounds in edible oils and each type of oil has its own characteristic TAG profile (Endo et al., 2011; Lerma-García et al., 2011). Currently, the IOC uses TAG quantification in combination with fatty acid analysis to detect adulteration. The official TAG analysis involves lengthy sample preparation followed by either high performance liquid chromatography (HPLC), or gas chromatography (GC) with direct on-column cold injection (IOC, 2001; IOC, 2013; IOC, 2018). For determination of fatty acids, additional sample preparation steps are required including careful methylation and recovery steps, prior to GC analysis (IOC, 2013; Liu, 1994). Nonetheless, TAG analysis alone has traditionally not been used for olive oil purity determination, in spite of its great potential in characterizing edible oils, because these compounds can be difficult to separate and analyze (Indelicato et al., 2017). Given these challenges, a method capable of specifically measuring TAGs with minimal sample preparation is highly attractive and can be used as a robust tool to detect adulteration in olive oil.

Traditional instruments used for TAG analysis include HPLC with refractive index (RI) detection or ultraviolet (UV) absorbance detection (Kiritsakis et al., 2002; Lee et al., 2001; Ruiz-Gutierrez & Barron, 1995) or GC with flame ionization detector (FID) (IOC, 2018). The challenge with these detectors is they often have low sensitivity and limited dynamic range for TAG quantification (Vehovec & Obreza, 2010). Specifically, UV detectors have low sensitivity for TAGs because TAGs containing the saturated palmitic and stearic fatty acids lack a strong chromophore and RI detectors are incompatible with the gradient separations necessary to

satisfactorily resolve TAG peaks. GC-FID approaches can be more sensitive but they are not direct and require extensive preparation such as esterification steps (Kail et al., 2012; Naviglio et al., 2017). Mass spectrometry (MS) has offered a more sensitive and specific method, which greatly improves analysis of TAGs (Cozzolino et al., 2010; Holčapek et al., 2005; Jakab et al., 2002; Jergovic et al., 2017; Luo et al., 2019; Mottram et al., 1997). However, a benchtop mass spectrometer is expensive to purchase and maintain and requires a specialist for operation. UHPLC coupled with CAD is highly suitable for TAG analysis and offers several benefits over other analytical approaches. CAD is universal (able to measure all non-volatile and many semi volatile analytes), offers uniform response (independent of chemical structure enabling single calibrant quantification when standards are unavailable), has a wide dynamic range, sub-ng sensitivity, gradient compatibility and is simple to operate (Gamache, 2017). Furthermore, analytes do not require a chromophore (e.g. UV) or form gas phase ions (e.g. MS) in order to be detected. In recent studies, UHPLC-CAD showed improved analyte resolution and analytical run time for TAG comparing to the IOC method (De la Mata-Espinosa et al., 2011; De la Mata-Espinosa et al., 2011; Lísa et al., 2007; Lucci et al., 2018).

In this study, a follow-up from the pilot proposal by Plante et al. (2014), we developed a fast, one-hour detection approach of extra virgin olive oil adulteration and tested with blind samples. Firstly, a UHPLC-CAD was employed to separate and analyze the TAGs in a variety of pure oil samples and oil blends after a simple oil dissolving in organic solvents. Once TAGs were separated, similarities and differences between different oils based on their TAG profiles were statistically evaluated by principal component analysis (PCA). PCA, a statistical technique used to visualize sample variation and highlights patterns in a data set, can be used to cluster different types of oils and was previously shown to be a viable method to determine olive oil

purity when combined with an analytical chemical method (Gómez-Caravaca et al., 2016; Lísa et al., 2009; Yang et al., 2013). Using TAG profiles to build a PCA to differentiate oils offers a time-efficient and cost-effective solution for detecting olive oil authenticity that can be easily utilized by the government officials and oil industries at various food fraud controlling points.

Materials and Methods

Chemicals and reagents

HPLC grade solvents (acetonitrile, chloroform, hexane, isopropanol, methanol and toluene) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Hydrochloric acid and anhydrous sodium sulfate were also obtained from Thermo Fisher Scientific. TAG standards were acquired from two vendors: 1,2-linolein-3-stearin (LLS), 1,2-linolein-3-olein (OLL), 1,2-olein-3-stearin (OOS), 1-palmitin-2-olein-3-linolein (POL), 1,2-palmitin-3-linolein (PPL) and 1-palmitin-2-stearin-3-olein (PSO) were purchased from Larodan (Solna, Sweden); trilinolein (LLL) and triolein (OOO) were obtained from MilliporeSigma. Nanopure water (18.2 MΩ-cm) was prepared with a Milli-Q system (Millipore, Bedford, MA, USA).

Oil samples

A total of 25 fresh EVOO samples were analyzed for this study. The samples were collected from various California counties and were all single-variety EVOO including 13 Arbequina, 6 Arbosana, and 6 Koroneiki oils. Other oil samples were purchased from various vendors. These samples included 11 grapeseed oils, 3 soybean oils, 7 canola oils, 4 high-oleic safflower oils, and 5 high-oleic sunflower oils. Prior to analysis, each oil was transferred to a 40 mL amber vial and stored in a dark fridge at 3ºC in order to minimize decomposition and changes in TAG profiles. Adulterated olive oil samples were prepared by blending an Arbequina

EVOO sample with each potential oil adulterant: grapeseed, soybean, canola, high-oleic sunflower, high-oleic safflower. Seven blends were made with the Arbequina EVOO and each adulterant, which covered a wide range in concentration from 95% EVOO with 5% of the adulterant to 10% EVOO and 90% of the adulterant. For method validation, a member of the lab prepared fifteen blind-sample blends of an Arbequina extra virgin olive oil with different adulterants, which were chosen from the adulterant oil categories listed above.

Triacylglycerol (TAG) analysis

The TAG analysis method was adopted and revised from Plante et al. (2014). In a 10 mL volumetric flask, 100 µL of olive oil was diluted with methanol/chloroform (50:50, v/v) to make the final concentration of 1% before being placed in a vial and loaded onto the Vanquish™ Flex UHPLC-CAD system (Thermo Fisher Scientific, Waltham, MA, USA) for analysis. The Vanquish™ Flex CAD is identical to the Corona Veo CAD. Analytes were separated on a Thermo Scientific™ Accucore™ C18 column (100 mm x 2.1 mm; 2.6 µm). The injection volume was 1 µL and the flow rate was 0.5 mL/min. Mobile phase A was acetonitrile and mobile phase B was isopropanol. The solvent gradient conditions were adapted from Plante et al. 2014 and were as follows: from Start, 10% B; 2 min, 10% B; 25 min, 40% B; 30 min, 60% B; 35 min, 90% B; 40 min, 50% B and 45 min 10% B. The sample chamber was maintained at 25 °C and the C18 column temperature was kept at 50 °C throughout the chromatographic sequence. The CAD evaporation temperature was set at 50 °C, with a power function of 1.00, filter of 5 s, and data collection rate of 10 Hz. The retention time of each individual peak from the chromatogram was compared against the retention time of available TAGs standards and previous literature (Jakab et al., 2002; Lísa and Holcapek, 2008; Lucci et al., 2018). The eleven TAGs used for analysis were chosen because they

either are the primary TAG components in EVOO (such as OOO) or were not present in EVOO at high concentrations, but are still important in distinguishing EVOO from other adulterant oils, such as LLL (IOC, 2001). Data acquisition and interpretation were performed using the Thermo Scientific™ Dionex™ Chromeleon™ 7.2.6 Chromatography Data System. Peak area ratios were used for statistical analysis. The ratios were calculated for the TAGs in each sample by comparing each peak area to the total TAG area. When using the CAD, the peak area ratios reflect the TAG abundance.

Statistical analysis

Statistical analysis was accomplished using Originlab Corporation software version "OriginPro 2016 Sr2." This program was used to run PCA on all samples analyzed with the UHPLC-CAD. The TAGs were used as the variables in these plots and the oil types as treatments. Confidence ellipses and principal component scores were computed by Originlab.

Fatty acid profile (FAP)

The International Olive Council (IOC) official method (COI/T.20/Doc. No 33/Rev.1 2017) was adopted for fatty acid profile analysis with modifications. Approximate 0.01 g of EVOO sample was weighed and dissolved in 0.4 mL of toluene using 30s of vortexing. Then 3 mL of methanol and 0.6 mL of methanol/HCl (80:20, v/v) were added, and the mixture was vortexed again for another 30s and kept at 80°C for one hour. The mixture was cooled to ambient temperature prior to mixing with 1.5 mL of hexane and 1 mL water. After the aqueous/organic mixture was completely separated into two phases, the upper layer, which contained the methyl esters, was decanted. An adequate amount of anhydrous sodium sulfate was added to remove water residue. The clear solution was then transferred into GC vials prior to analysis. The GC-

flame ionization detector (FID) analysis was conducted on a Varian 450-GC (Agilent Technologies, Santa Clara, CA, USA) equipped with an 8400 auto-sampler and a 1177 split/splitless Siltek® coated injector. The injection volume was 0.2 uL. Helium was used as carrier gas at a flow rate of 1.5 mL/min. A 60 m x 0.25 mm x 0.25 um DB-23 capillary column (Agilent Technologies, Santa Clara, CA, USA) was used to achieve the separation of individual fatty acid compositions. The injector temperature was held at 270 °C at a split ratio of 100. The GC oven program was initially held isothermally at 100 °C for 8 min; then ramped at 6.5 °C/min to 170 °C followed by ramping at 2.7 °C/min to 215 °C and held for 12 min; finally ramped at 15 °C/min and held for 10 min to bake off remaining high-boiling residues. The FID temperature was 280 °C. The detector gas consisted of hydrogen (flow rate: 30 mL/ min), air (flow rate: 300 mL/min), and helium make up gas (flow rate: 25 mL/min). A FAME reference standard mix was used for peak identification by retention time.

Results and Discussion

UHPLC-CAD method validation

The UHPLC-CAD system was used to determine the TAG profiles for this study. Two TAGs, LLL and OOO, were used to assess the method performance and variability. These TAGs were chosen because LLL is a TAG of low concentration in EVOO while OOO is one of highest concentration TAGs in olive oil. Three replicates were used to determine the inter-day and intra-day retention time and peak area variability for these two TAGs, Table 6.1. The limit of detection was determined with a low-level standard, 0.5 µg/mL, for both LLL (signal to noise ratio of 3.25) and OOO (signal to noise ratio of 4.33). LLL had a detection limit of 0.46 µg/mL and OOO 0.35 µg/mL, which was determined using a signal to noise ratio of 3:1. The limit of

quantification was calculated with the same 0.5 µg/mL concentration standard using a signal to noise ratio of 10:1 and was found to be 1.54 µg/mL for LLL and 1.15 µg/mL for OOO.

Determination of oil clusters using PCA

The TAG profiles were determined for olive oil (Figure 6.1) as well as for five common olive oil adulterants including high-oleic sunflower, high-oleic safflower, canola, soybean, and grapeseed oils. These oils were chosen based on the likelihood of use as adulterants in EVOO. In addition, high-oleic sunflower and high-oleic safflower oils were selected due to their high similarity in TAG composition compared to olive oil (Chiavaro et al., 2009). Unlike normal sunflower and safflower oils, high-oleic oils have less LLL and more OOO TAGs, making them more similar in composition to EVOO and thus likely candidates for adulteration (Chiavaro et al., 2009).

The percentage of each TAG relative to the total TAG area was quantified for each oil sample. These eleven TAGs were then used as the variables for PCA. The biplot obtained from the two principal components, representing the eleven TAGs quantified for each oil and the distinct clustering for each oil type is shown in Figure 6.2. These two principal components account for 81.9% of the variance among the seven oil samples. Olive oil is positively correlated with OOP, PSO, and PPS/PPO TAGs, while high-oleic sunflower and high-oleic safflower are correlated with OOO, OOS, and OOL. Soybean oil is best correlated with LLS/POL and PPL TAGs. Grapeseed oil has a distinct cluster on the far right of the plot along the PC2 axis. Canola oil also forms a distinct cluster in the lower right quadrant. The 95% confidence ellipse around the fresh olive oils indicates that olive oil also groups well in this biplot and does not overlap with any other oil type. The distinctness of the olive oil cluster in this plot shows this approach's

potential use in detecting adulteration of EVOO with any of the oils used in this study (see Figure 6.2).

Emulating adulteration with EVOO blends

Figure 6.2 demonstrates that the approach can adequately separate olive oil from other common oil adulterants using PCA. However, to use this approach for detecting adulteration it is necessary to ensure PCA can separate EVOO from blends with each oil type. In order to address this, blends were made with EVOO and each oil type in Figure 6.2, from 95% EVOO with 5% of the adulterant to 10% EVOO and 90% of the adulterant. Each of these blends were then analyzed using PCA. High-oleic sunflower is shown in Figure 6.3 as an example. The biplot in Figure 6.3 accounts for 93.4% of the variation between the samples. PC1 accounts for most of the variation between high-oleic sunflower and EVOO, with EVOO clustered on the right side of the plot and the high-oleic sunflower samples on the left. The blended samples containing the most EVOO, such as the 95% sample, are closest to the EVOO cluster, while those containing the least EVOO (e.g., the more high-oleic sunflower oil blend), is furthest away. Figure 6.3 shows that using TAG profiles in combination with PCA can differentiate EVOO from high-oleic sunflower oil at adulteration levels greater than 10%. While it is possible that adulteration could occur at levels around 10% or below, it is very uncommon as it is no longer cost-effective for the companies that seek economic advantages.

Optimizing separation of adulterated oils in PCA

In an effort to better separate the pure EVOO cluster from the blended samples, the number of TAGs used in each plot was decreased. When differentiating many oils, as in Figure 6.2, all of the TAGs are necessary to include, as each oil is characterized by different TAG

profile. However, Figure 6.3 shows high-oleic sunflower oil is characterized by LLL, OOO, and OOS primarily. By choosing only TAGs that are best correlated with the adulterant and contribute the most to the variance between the adulterant and EVOO, optimal separation between each adulterant and EVOO can be achieved. The biplot in Figure 6.4 shows a significant improvement of separation using only three TAGs with PC1 and PC2 now accounting for 98.7% of sample variation, as opposed to 93.4% when using all of the TAGs. Now, with only using LLL, OOO, and OOS TAGs, as shown in Figure 6.4, the EVOO cluster is tightened significantly and an adulterated EVOO sample with high-oleic sunflower can be distinguished at 5%.

The process of eliminating TAGs to gain optimum separation between EVOO and the adulterant was performed for all oil listed in section 2.2. For each potential adulterant, biplots were produced (Figures S6.1-S6.4). From these plots, it was determined that adulteration of EVOO could be distinguished at a level of 5% for grapeseed and high-oleic sunflower oils and 10% for canola, soybean, and high-oleic safflower oils. It should be noted that for each oil the best separation was achieved using three TAGs with LLL always being one of the three. Olive oil has a much lower linoleic fatty acid content than many other oils (Jakab et al., 2002), thus LLL is an important TAG that needs to be used to distinguish EVOO from other oils types. It is necessary to use an instrument like the UHPLC-CAD that enables the separation and quantification of LLL, as well as other linoleic acid containing TAGs such as OLL and LLP. Because this approach relies on the placement of each sample on the PCA plot, it is possible this separation would be less obvious if an EVOO was adulterated with an oil that was closer to the EVOO cluster in Figure 6.4. Therefore, blends were made with another high-oleic sunflower oil that was more correlated with OOS and OOO (as opposed to LLL) and was closer in proximity to EVOO on the PCA in Figure 6.4. The separation was maintained with this sample and the

approach was still able to distinguish EVOO adulteration with high-oleic sunflower oils at 5% adulteration.

Method validation and implementation

If a specific adulterant is expected, then the sample can easily be analyzed using a PCA plot like Figure 6.4 and can be used to determine the adulteration percentage in each oil type. However, if an adulterant is not known, then this approach could be implemented in a step-wise fashion to determine not only the adulterant, but also the level of adulteration. Step one: Figure 6.2 can be used to initially scan for purity of olive oil from many different potential adulterants. Based on the oil's location the potential adulterant can be predicted. Step two: The oil can be analyzed using a plot like Figure 6.4 to determine the level of adulteration of the sample. This stepwise approach is easy for industry and customs to follow and does not require a trained specialist.

To demonstrate the implementation of this approach, blind samples were tested using the step-wise fashion described above. Table 6.2 summarizes the results of true and predicted values for 15 blind samples. Sample Blind 1 was plotted on Figure 6.2 to generate Figure 6.5a. It was predicted that this sample was adulterated with canola oil based on the sample's alignment between the EVOO and canola oil cluster and that its TAG profile was best correlated with OOP, PSO and PPS/PPO. Figure 6.5b shows "Blind 1" on the optimized biplot containing only EVOO and canola oils. Blind 1's location directly next to the 80% EVOO points, indicates it is adulterated with approximately 20% canola oil. It was later confirmed that "Blind 1" was adulterated with canola oil at a level of 20% (Table 6.2).

Another example of this step-wise process is demonstrated with sample “Blind 15.” It is clear on Figure 6.6a (generated from Figure 6.2) that Blind 15 is well correlated with OOS and OOO and is likely adulterated with high oleic sunflower or safflower oil. However, it is difficult to predict which of these oils is the adulterant as the sample is located in the middle of the two clusters. Upon moving to the secondary plots Figure 6.6b, it is apparent that high oleic sunflower oil is the correct adulterant as Blind 15 is aligned with the other EVOO and sunflower blends. It was then correctly predicted that Blind 15 was adulterated with 85-90% high oleic sunflower oil (Table 6.2).

Blind 15 reveals that with the current PCA model it can be difficult to distinguish the high oleic sunflower and safflower clusters, particularly if the adulteration is at a very high or low percentages. Table 6.2 shows that although the percent of adulteration could be determined for samples Blind 10 and Blind 13, the adulterant could not be confidently confirmed. This is likely due to the small sample size for the adulterant oils in the current model, which leads to oil clusters on the PCA that are not well-defined. If the sample size for each of the adulterant oils were to be increased, it is expected that the clusters for these adulterant oils will be clearer, which will greatly increase the correct prediction rate. The more samples that are analyzed the better the TAG variation within each oil type is accounted for. Nonetheless, Table 6.2 shows that for 12 of the 15 blind samples the adulterant was predicted correctly, and the percent of adulteration was predicted correctly for all blind samples within 10 percent.

When considering widespread implementation of this method, it is worth noting that this study only used olive oils from California. The TAGs present in an olive oil change depending on its fatty acid profile. The range of the fatty acid profiles for the oils used in this study are

listed in Table S6.1. The applicability of this study to the global olive oil industry can currently only be ensured for olive oils that are within the fatty acid range of the oils used in this study.

Conclusions

In this study, the usefulness and accuracy of a step-wise PCA approach for EVOO adulteration detection using TAGs detection with UHPLC-CAD is demonstrated. This approach has no sample preparation and enables analyses to be completed with less waste and less cost compared to traditional methods, making it feasible for industries and government officials to be able to adopt this as a screening tool to quickly identify any mislabeled EVOO and reject them on the spot. This kind of fast identification tool that does not require specialized trained chemists and prevents fraudulent products from being imported or exported during critical points, is urgently needed. For future work, we aim to implement this approach for widespread use by expanding the TAG database with more oil types, more samples within each type, multiple adulterants, oils made from different cultivars, geographical locations, climate and such to better define clusters in the PCA thereby improving the accuracy of this approach.

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Conflict of Interest Statement

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Tables

Table 6.1: Summary of method validation data for the UHPLC-CAD.

TAG	Recovery[1]	Intra-day Variability[2]		Inter-day Variability[2]		LOD	LOQ	R^2 (0.005-2.5 mg/mL)
	%	Retention time (% RSD)	Peak area (% RSD)	Retention time (% RSD)	Peak area (% RSD)	µg/mL	µg/mL	
LLL	104.5	0.139	0.600	0.698	2.324	0.46	1.54	0.992, y=11.02x + 1.21,
OOO	96.9	0.177	0.258	0.599	5.062	0.35	1.15	0.992, y=24.38x + 2.73

[1]Recovery was determined with spike experiments. Spike amount was approximately equal to amount of LLL/OOO native in the olive oil sample.

[2]Percent relative standard deviations (% RSDs) values for both inter and intra-day variability experiments were determined using three injections.

Table 6.2: Summary of true and predicted values for the 15 blind samples. HO = high oleic.

Blind Samples	**True Values**		**Predicted Values**	
	Adulterant	% EVOO	Adulterant	% EVOO
1	Canola	80	Canola	80
2	Canola	45	Canola	40-45
3	Canola	15	Canola	10-15
4	Soybean	80	Soybean	75-80
5	Soybean	45	Soybean	35-45
6	Soybean	15	Soybean	<10
7	Grapeseed	80	Grapeseed	80
8	Grapeseed	45	Grapeseed	40-45
9	Grapeseed	15	Grapeseed	20
10	HO Safflower	80	Unclear[1]	75-80
11	HO Safflower	45	HO Safflower	50
12	HO Safflower	15	HO Safflower	20-25
13	HO Sunflower	80	Unclear[1]	80-85
14	HO Sunflower	45	HO Sunflower	40-45
15	HO Sunflower	15	HO Sunflower[2]	15

1Adulterant could not be confidently predicted as HO safflower or HO sunflower, however, the percent of adulteration determined was predicted correctly.

2Adulterant predicted as HO Sunflower or HO Safflower on Figure 6.2 and confirmed as the correct adulterant on the secondary plots.

Figure

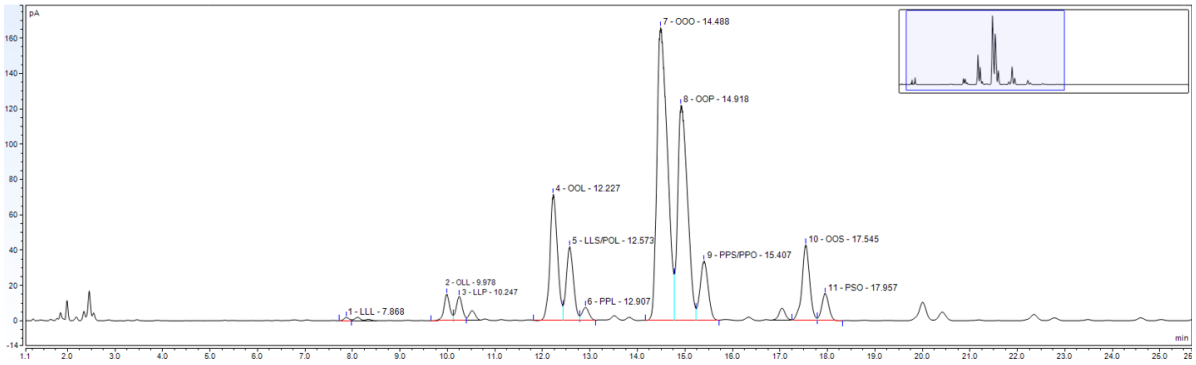

Figure 6.1. Chromatogram from UHPLC-CAD showing the eleven identified TAGs with corresponding retention times in a single-varietal Koroneki extra virgin olive oil sample.

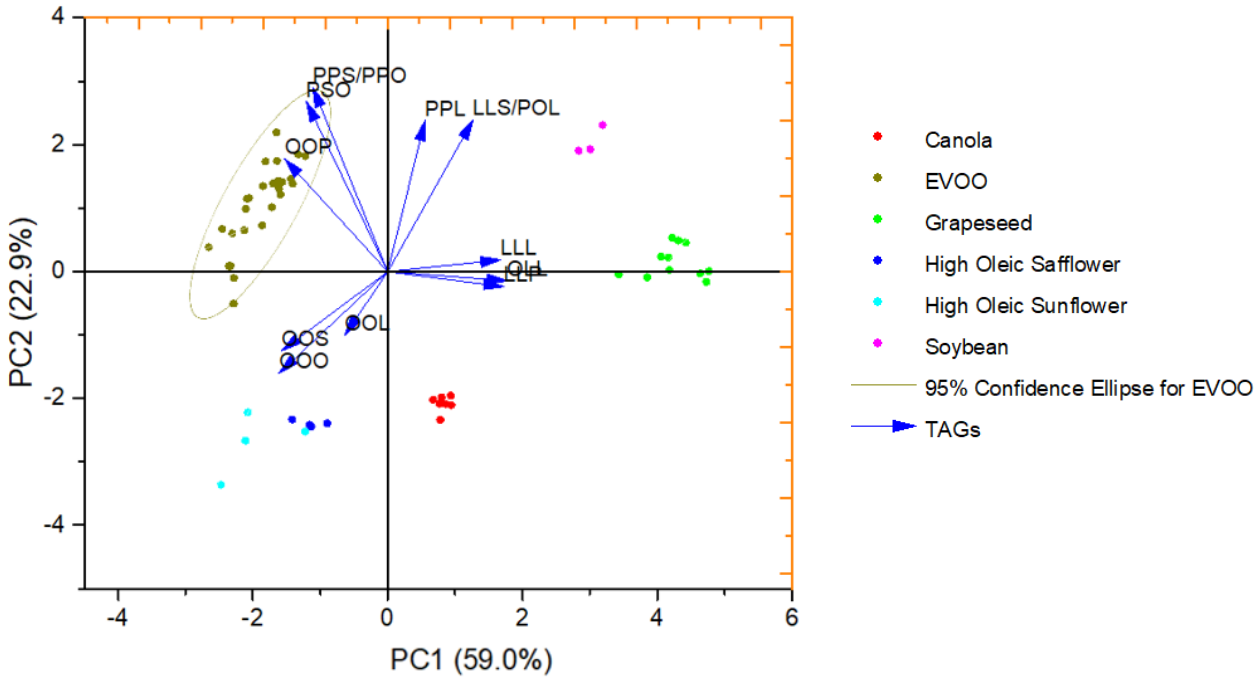

Figure 6.2. Biplot from the first and second principal components of PCA. Variables: eleven different TAGs quantified in each oil sample. Treatments: six different edible oils including extra virgin olive oil (EVOO). The ellipse is the 95% confidence ellipse for EVOO.

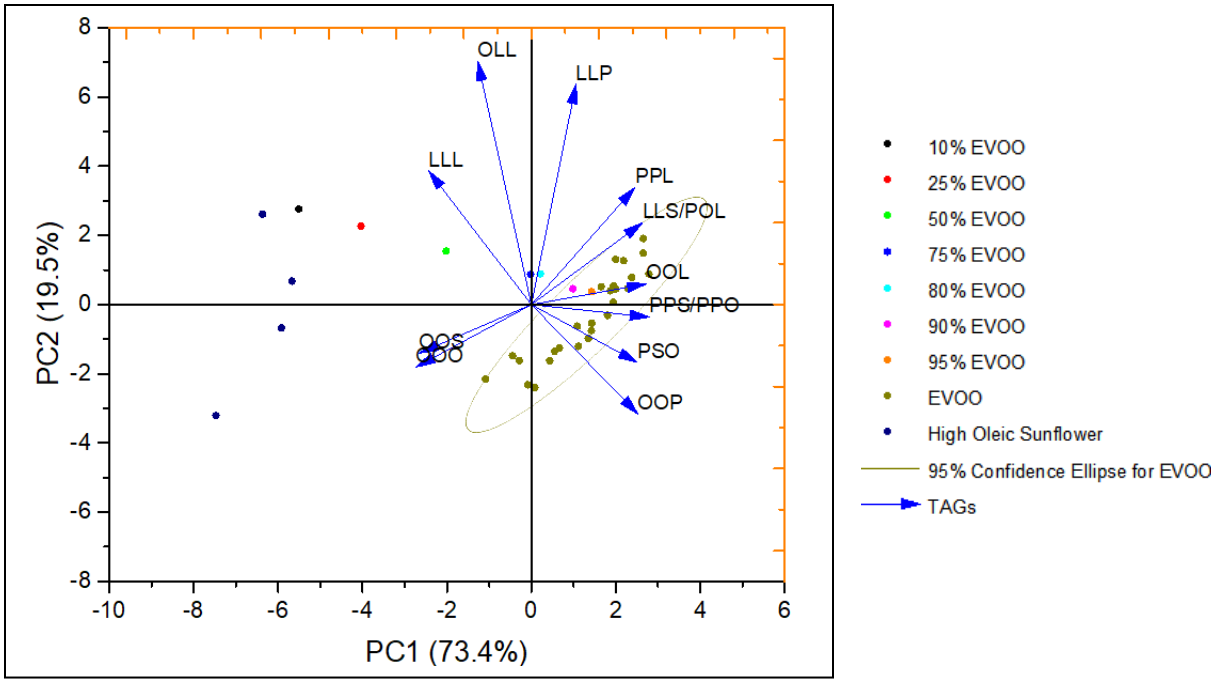

Figure 6.3. Biplot from the first and second principal components of PCA of high oleic sunflower oils and sunflower oil/EVOO blends. Variables: eleven different TAGs quantified in each oil sample. Treatments: pure EVOO, pure high oleic sunflower oils, and blends of high oleic sunflower oil and EVOO from 95% EVOO to 10% EVOO.

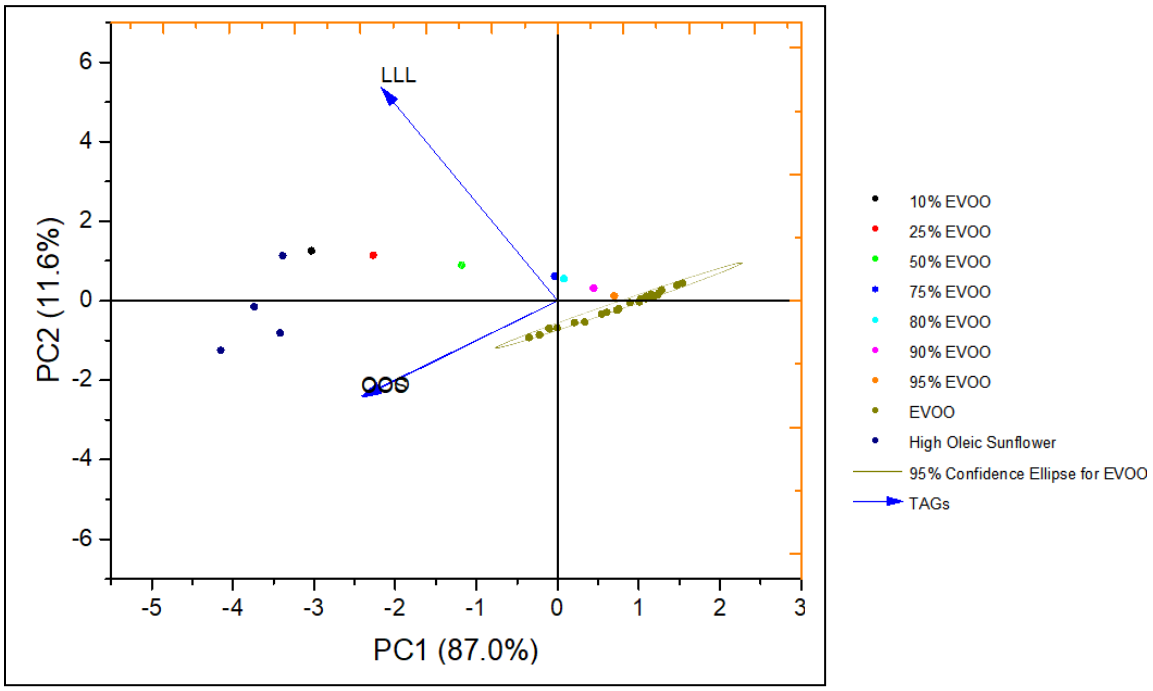

Figure 6.4. Biplot from the first and second principal components of PCA of high oleic sunflower oils and sunflower oil/EVOO blends. Variables: three different TAGs quantified in each oil sample. Treatments: pure EVOO, pure high oleic sunflower oils, and blends of high oleic sunflower oil and EVOO from 95% EVOO to 10% EVOO.

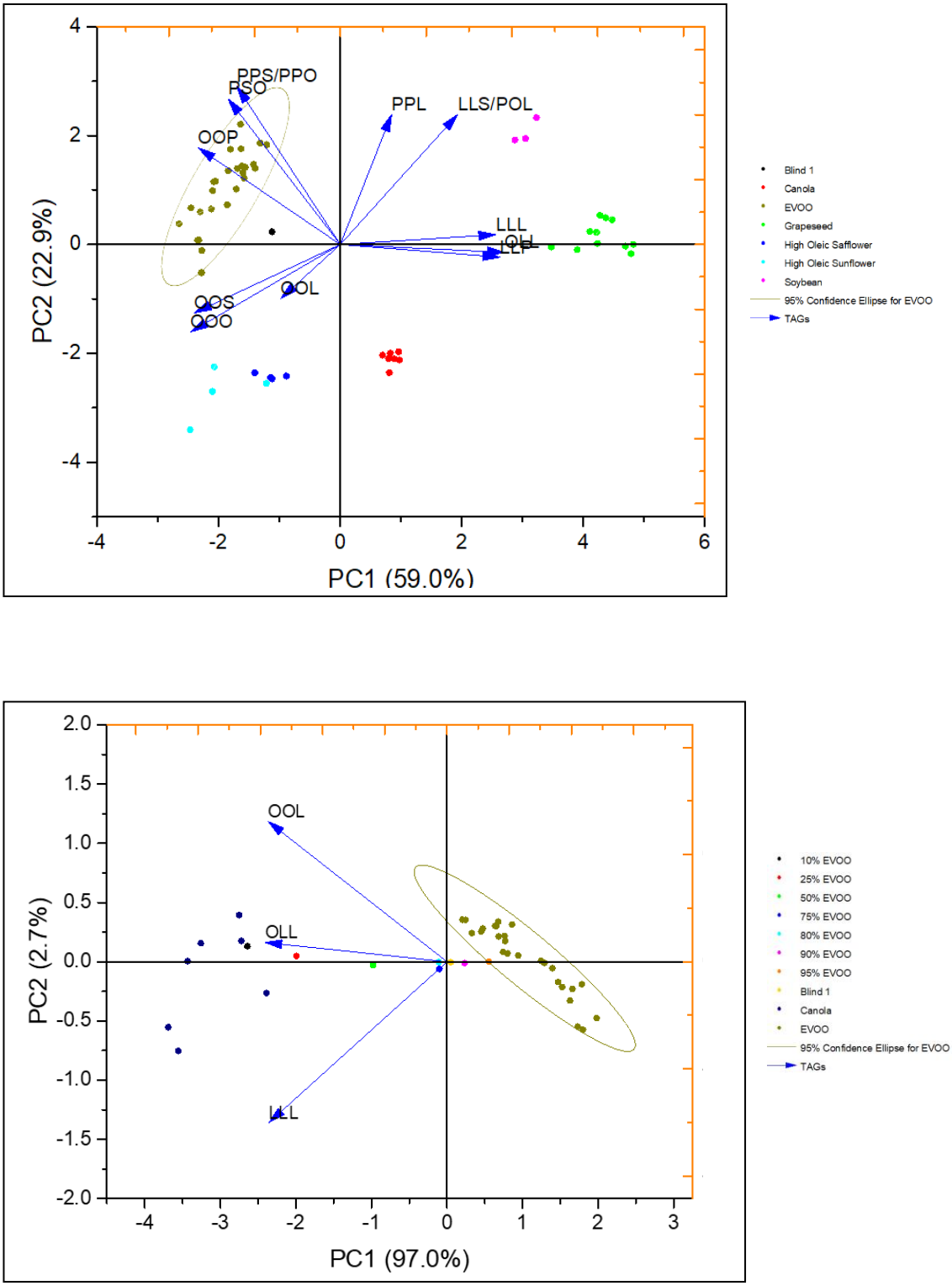

Figure 6.5. a) Biplot from the first and second principal components of PCA. Variables: eleven different TAGs quantified in each oil sample. Treatments: six different edible oils and a sample

of unknown composition, “Blind 1”. The ellipse is the 95% confidence ellipse for EVOO; b) Biplot from the first and second principal components of PCA for canola oils and blends with EVOO. Variables: three different TAGs quantified in each oil sample. Treatments: pure EVOO, pure canola oils, blends of canola oil and EVOO from 95% EVOO to 10% EVOO and a sample with an unknown blend percent, Blind 1.

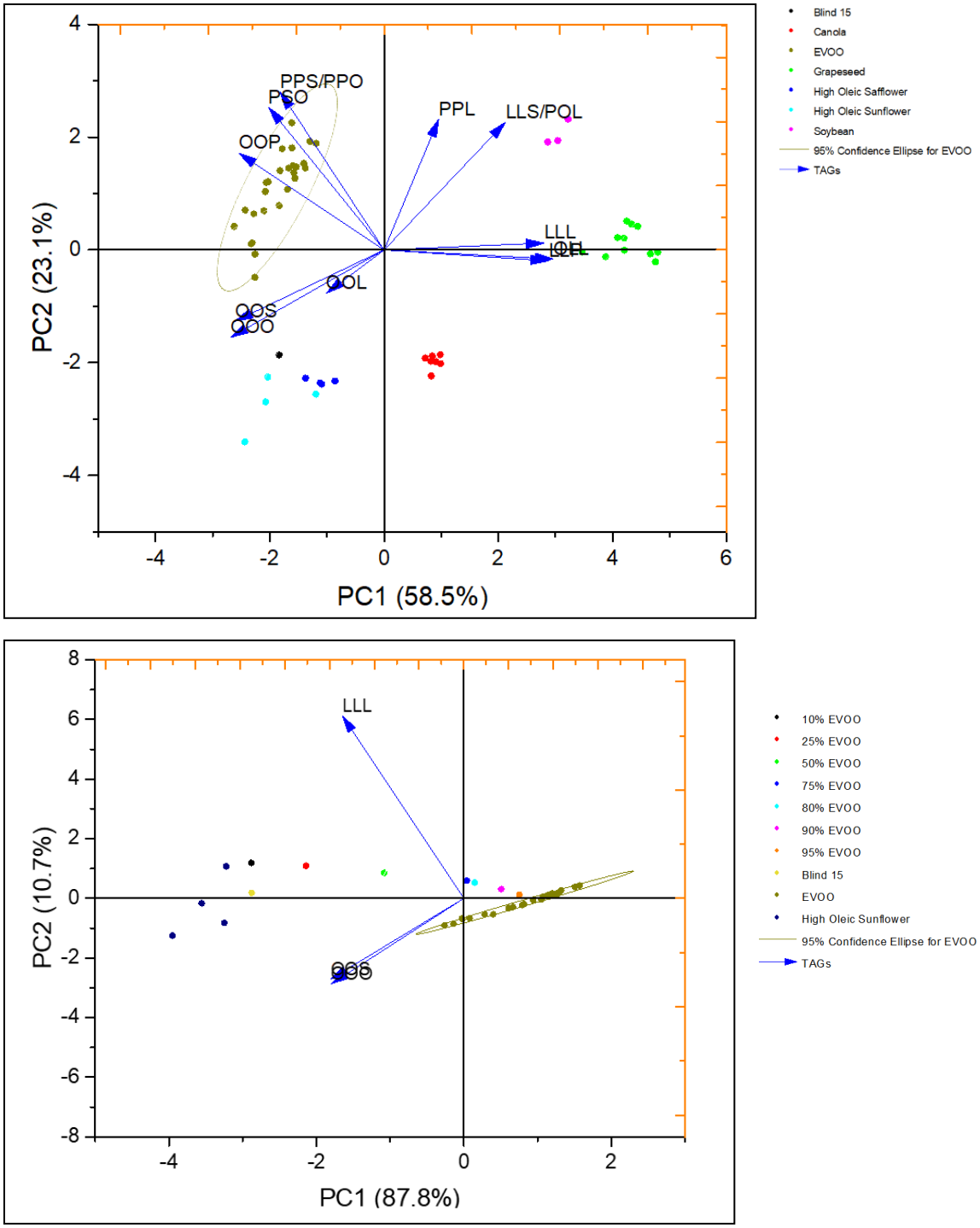

Figure 6.6. a) Biplot from the first and second principal components of PCA. Variables: eleven different TAGs quantified in each oil sample. Treatments: six different edible oils and a sample of unknown composition, “Blind 15.” The ellipse is the 95% confidence ellipse for EVOO; b)

Biplot from the first and second principal components of PCA for high oleic safflower oils and blends with EVOO. Variables: three different TAGs quantified in each oil sample. Treatments: pure EVOO, pure canola oils, blends of high oleic safflower oil and EVOO from 95% EVOO to 10% EVOO and a sample with an unknown blend percent, Blind 15.

Supplemental Tables

Table S6.1: Fatty acid range for the olive oils used in this study.

Fatty Acid	Percent (%)
Palmitic Acid (C16:0)	13.1-17.9
Palmitoleic Acid (C16:1)	0.6-2.4
Heptadecanoic Acid (C17:0)	0-0.2
Heptadecenoic Acid (C17:1)	0.1-0.3
Stearic Acid (C18:0)	1.4-2.6
Oleic Acid (C18:1)	61.3-79.1
Linoleic Acid (C18:2)	4.8-14.0
Linolenic Acid (C18:3)	0.4-0.9
Arachidic Acid (C20:0)	0.2-0.4
Gadoleic Acid (Eicosenoic) (C20:1)	0.2-0.3
Behenic Acid (C22:0)	0-0.1
Lignoceric Acid (C24:0)	0-0.1

Supplemental Figures

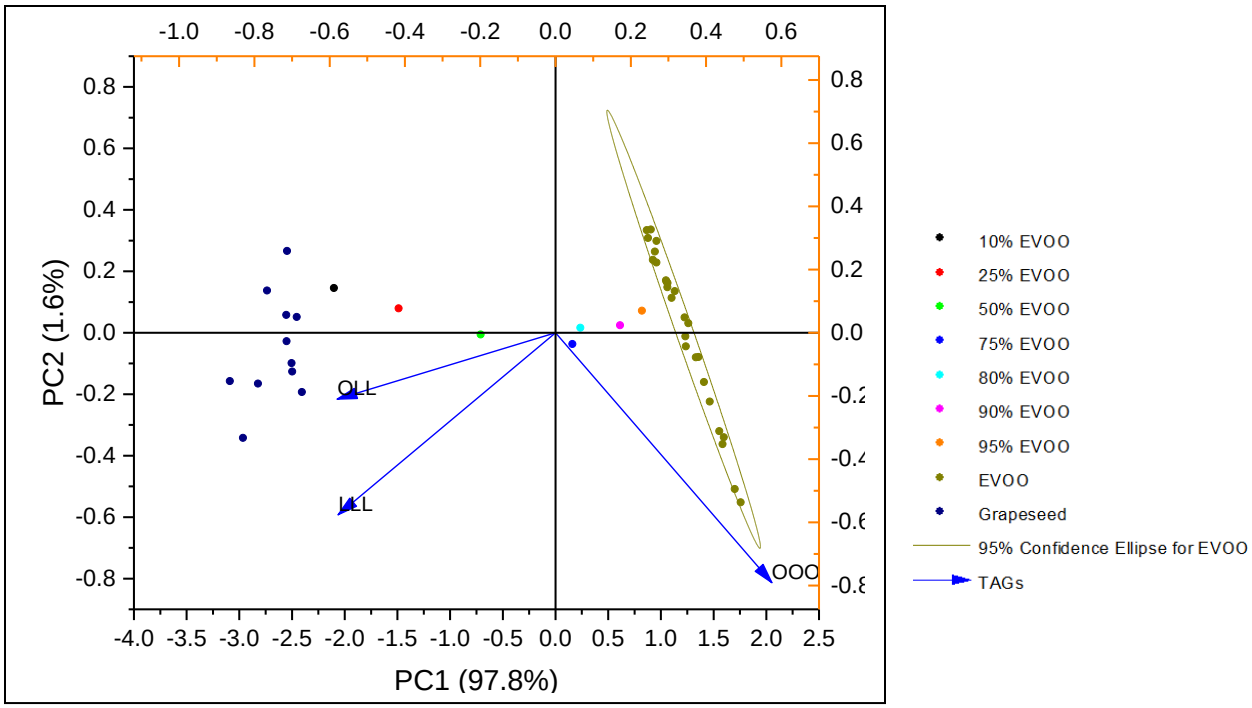

Figure S6.1. Biplot from the first and second principal components of PCA for grapeseed oils and blends with EVOO. Variables: three different TAGs quantified in each oil sample. Treatments: pure EVOO, pure grapeseed oils, and blends of grapeseed oil and EVOO from 95% EVOO to 10% EVOO. The ellipse is the 95% confidence ellipse for EVOO.

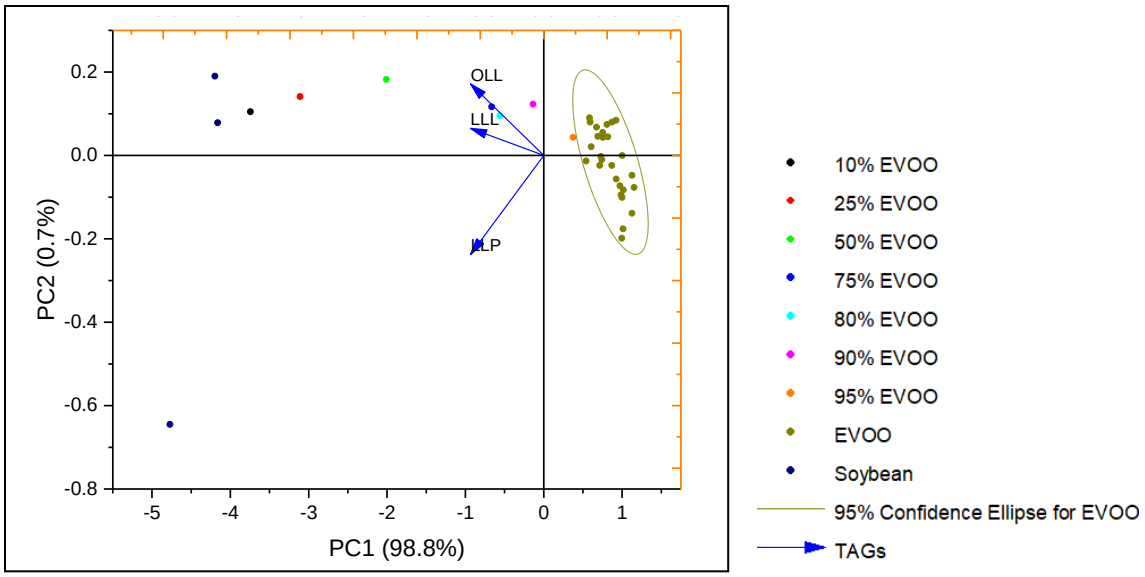

Figure S6.2. Biplot from the first and second principal components of PCA for soybean oils and blends with EVOO. Variables: three different TAGs quantified in each oil sample. Treatments: pure EVOO, pure soybean oils, and blends of soybean oil and EVOO from 95% EVOO to 10% EVOO.

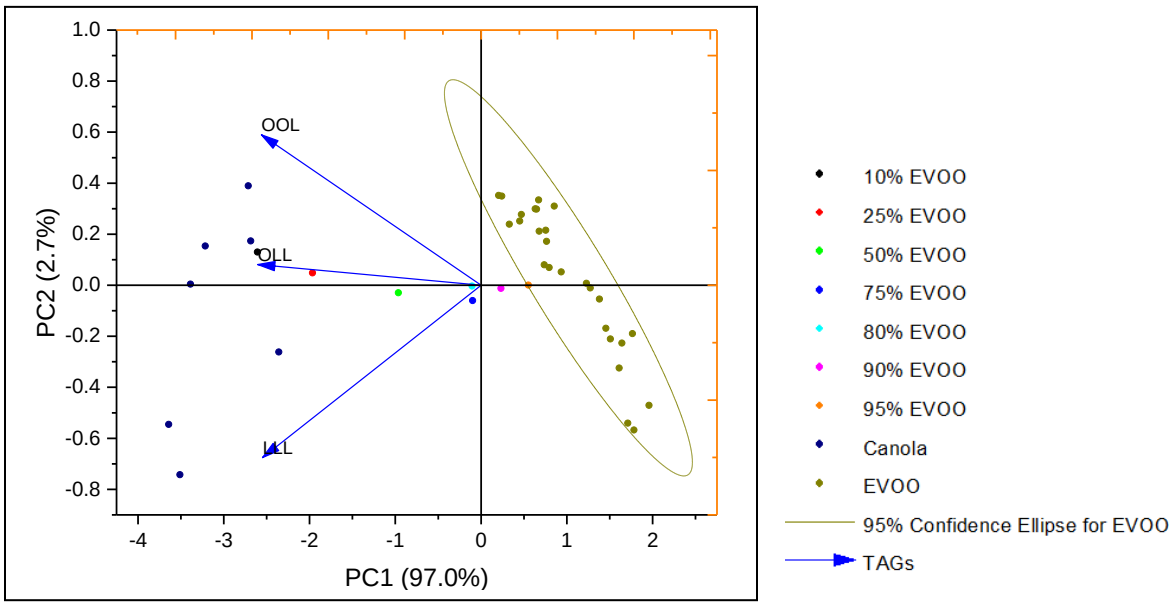

Figure S6.3. Biplot from the first and second principal components of PCA for canola oils and blends with EVOO. Variables: three different TAGs quantified in each oil sample. Treatments: pure EVOO, pure canola oils, and blends of canola oil and EVOO from 95% EVOO to 10% EVOO.

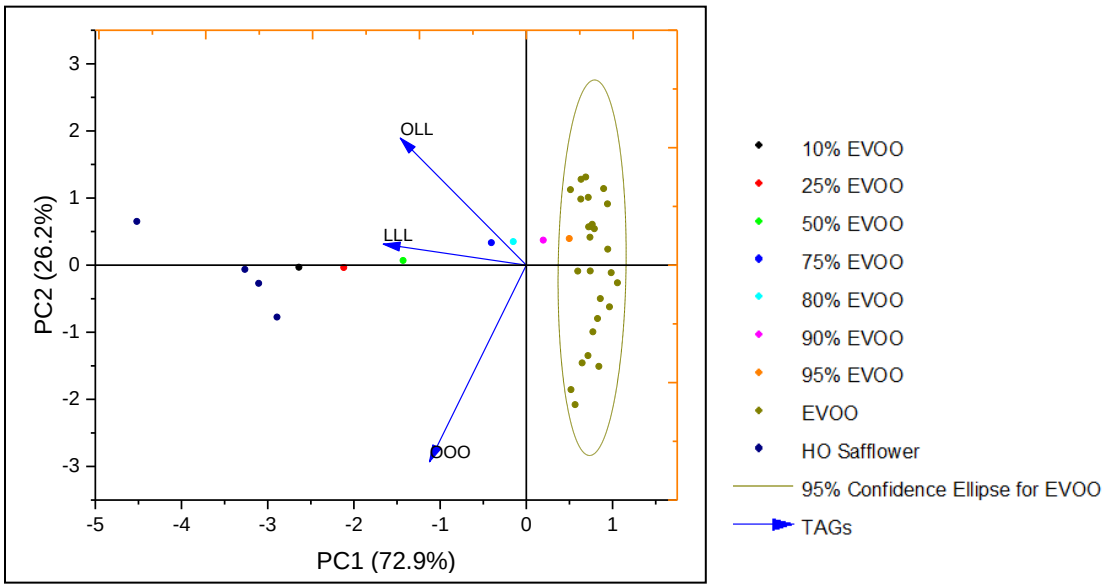

Figure S6.4. Biplot from the first and second principal components of PCA for high-oleic safflower oils and blends with EVOO. Variables: three different TAGs quantified in each oil sample. Treatments: pure EVOO, pure high-oleic safflower oils, and blends of high-oleic safflower oil and EVOO from 95% EVOO to 10% EVOO.

Chapter 7

Tandem triacylglycerol (TAG) and PCA adulteration detection approach for avocado oil

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Abstract

Traditional methods used to determine oil purity like fatty acids and sterols are time consuming and chemically wasteful; standards that utilize these methods require a large set of samples to cover natural variables to establish upper and/or lower limits for each compound. Due to this, it can be challenging to determine the purity of newer products on the market, like avocado oil, when standards have not yet been fully developed. Triacylglycerol analysis in tandem with principal component analysis differs from these tradition methods; standard ranges for each triacylglycerol are not needed to determine purity. This study built on our earlier work on olive oil but instead was developed for avocado oil using oils accounting for a wide range of natural variables to measure avocado oil triacylglycerols and apply PCA (principal component analysis) to detect adulteration in avocado oil. This method had the same purity determination accuracy as traditional fatty acid and sterols methods, while being less time consuming, producing less

chemical waste and being easier to perform than the original methods with the added advantage that it can be utilized immediately by industry while official standards are still being developed.

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Background

There is an urgent need to detect adulterations in avocado oil. Standard development is underway, primarily by CODEX Alimentarius, an international food standards and regulation agency. However, this process takes time and significant data input from industry members of producing countries, academics, and the government. Fatty acid profile (FAP) and sterols are currently the two purity parameters being included in CODEX standards on fats and oils (CODEX, 2021), with each of their own advantages and disadvantages. Sterols requires significantly more sample prep than FAP but is often seen as a necessary addition to FAP as some adulterated samples can pass as pure with only fatty acids, and vice versa.

In 2020, our research group analyzed 22 avocado oils on the market in the US to evaluate their quality (e.g., free fatty acidity, peroxide value, UV absorbances, vitamin E) and purity (e.g., fatty acids, sterols, triacylglycerols) and found quality issues and adulterations, particularly with seed oils, in many of the samples (Green and Wang, 2020b). Aside from the typical, standard tests, other methods have been developed to detect the presence of seed oils in avocado oil. Fourier transform infrared spectroscopy (FTIR) has been the most popular analytical tool for adulteration detection in avocado oil and has been used in multiple studies including Quinones-Islas et al., 2013 and Jimenez-Sotelo et al., 2016 where it was combined with SIMCA and partial least squares analysis (PLS) to detect the presence of sunflower, soybean, and canola oils in avocado oils. Lumakso et al., 2015 also utilized FTIR with PLS and to differentiate avocado oil from grapeseed and sesame oil, while Rohman et al., 2016 used it with PLS and principal component regression (PCR). These FTIR applications have the of advantage of being fast with no sample pretreatment, however, some of the chemometric approaches could be difficult to implement in a wide-scale industrial setting. In addition, for

method prediction to be accurate it is necessary to have a breadth of samples encompassing how avocado oil varies with time, region, and other variables and it has not yet been assessed if these methods have the same level of accuracy when using this breadth of samples. Nuclear magnetic resonance (NMR) in Teng et al. 2021 and Jin et al. 2022 can also be used as an adulteration detection tool. Like FTIR, NMR also requires minimal to no sample preparation. It can be seen as a more rapid approach to detecting fatty acids, which are already used in purity determination. It has the added advantage that it could also detect minor components and oil quality with one analysis, however, cost and sample variety is a barrier to wide-spread use.

Since our first study, we have been working on collecting FAP and sterols of authentic avocado oil samples as these methods require an acceptable range for each fatty acid and sterol to be developed for avocado oil standards. At the same time, the rapid method our group developed using triacylglycerols in combination with multivariate analysis to detect adulteration in olive oil (Green et al., 2020a) has significant potential for the avocado oil industry. In brief, triacylglycerols (TAGs) are the primary constituents that make up oils. Determining purity directly using these compounds is convenient because the resources and time for sample prep is minimized. By using principal component analysis (PCA) to analyze the TAGs, a sample can simply be put on the PCA plot, and its purity can be determined by visualization. Comparing to FAP or sterols, this eliminates the need to establish upper and/or lower limits for individual fatty acid or sterol and is more accessible for personnel who may not be familiar with the fatty acid and sterol ranges of avocado oil and common adulterants. This study applies our previously developed method for olive oil, with some modifications, in a way that can directly and immediately benefit the avocado oil industry while acceptable FAP and sterols ranges are still determined. In addition, it can continue to be used as a screening tool for

adulteration in the place of fatty acids and sterols to reduce resources used by with the traditional purity methods.

Materials and Methods

Oil samples

Authentic avocado oils and potential avocado oil adulterants were used in this study. The potential adulterant oils included high oleic sunflower, high oleic safflower, soybean, and canola oil. Ten samples from each adulterant oil were purchased from grocery stores and online, were confirmed to be pure via fatty acid profile, then used for this study. The pure avocado samples (n=68) used in this study were previously described in Green and Wang, 2022a. Briefly, two different experimental designs were used to account for a variety of natural factors that can impact the chemical composition of avocado oil. The first design included region harvested (California and Mexico), harvest time, grade of fruit used, and using whole fruit or flesh to make the oil. These oils were cold-pressed and extracted mechanically. The second design accounted for region harvested (two locations within California), harvest time, cultivar, and using whole fruit or flesh to make oil. The oils from this design were solvent extracted using hexane. Test samples used for this method were pure oils sent from producers in different countries (n=3) and oils of unknown purity purchased from different regions in the US (n=25).

TAG analysis

The TAG analysis method was used as described in Green et al. 2020a. In brief, oil samples were diluted by 100 with methanol/chloroform (50:50, v/v) to make the final concentration of 1% before being placed in a vial and loaded onto the Vanquish™ Flex UHPLC-CAD system (Thermo Fisher Scientific, Waltham, MA, USA) using a Thermo Scientific™ Accucore™ C18 column (100 mm × 2.1 mm; 2.6 μm) for analysis. The injection volume was

1 μL and the flow rate was 0.5 mL/min. Mobile phase A was acetonitrile and mobile phase B was isopropanol. The solvent gradient conditions were: from Start, 10% B; 2 min, 10% B; 25 min, 40% B; 30 min, 60% B; 35 min, 90% B; 40 min, 50% B and 45 min 10% B. The sample chamber was maintained at 25 °C and column temperature at 50 °C. Thirteen TAGs were chosen because they either are the primary components in avocado oil or were important for distinguishing avocado oil from other potential adulterant oils. Peaks were identified with analytical standards and by comparing to previous samples analyzed with this method. Peak area ratios were used for statistical analysis. The ratios were calculated for the TAGs in each sample by comparing each peak area to the total TAG area. All samples were analyzed in duplicate.

Fatty acid profile

The fatty acid profile analysis was done as described in Green and Wang 2022a and according to the IOC official method for the determination of the fatty acid methyl esters by gas chromatography (COI/T.20/ Doc. No 33/Rev.1, 2017). In brief, approximately 20uL of oil was mixed with 3 mL of hexane. Then 200 uL of 2M methanolic KOH was added and vigorously mixed for 1 min. Samples were left until the top solution was clear and the organic layer was filtered using 0.2-micron PTFE filters. Samples were analyzed in duplicate. The GC-FID analysis was conducted on an Agilent 7890A GC using a 90 m × 250 μm × 0.25 μm DB-FastFAME capillary column. The injection volume was 1.0 μL and helium was used as a carrier gas at a flow rate of 1.9 mL min^{-1}. The injector temperature was held at 260 °C at a split ratio of 30. The GC oven program was initially held at 35°C for 1 min; then ramped at 65°C min^{-1} to 200 °C and held for 14 min, followed by a ramp of at 2.5°C min^{-1} to 210 °C, which was held for 5 min. The last ramp was at 12°C min^{-1} to 230°C and held for 20 min, giving a total run time of 49.2 min. The FID temperature was 260°C. The detector gas consisted of hydrogen (flow rate:

40 mL min^{-1}), air (flow rate: 400 mL min^{-1}), and helium make up gas (flow rate: 25 mL min^{-1}). Peak identification was performed using a FAME 37-component reference standard mix (MilliporeSigma).

Sterols profile

Sterols content was analyzed as described in Green et al. 2022a. The unsaponifiable fraction was prepared by drying 20 uL of internal standard 0.2% α-cholestanol ethyl acetate solution before adding 200 mg oil followed by 1.5mL of 2M KOH in 95% ethanol. The mixture was heated at 80 ºC for 25 min, mixed and heated for a second 25 min. Then, 13.5 mL DI water was added before loading onto a Phenomenex Strata DE SLE cartridge, 60cc tube, followed by two 1 mL rinses. The extract was eluted with five, 15 mL portions of diethyl ether after 15 min. Eluent was passed through a syringe packed with sodium sulfate then dried using a rotary evaporator and placed in an oven at 100 ºC for 10 min to remove remaining water. Dried extracts were reconstituted with 5 mL hexane. Next, the silica SPE columns (6 mL, 1 g sorbent, Agilent brand) were conditioned using two, 6 mL hexane rinses followed by 1 mL of 0.2M KOH in 98% ethanol, followed by a 5 mL hexane rinse. Each sample was then loaded onto the SPE cartridge then the cartridges were washed with 85 mL of hexane: diethyl ether (98:2) at 2 mL/min. The sterols fraction was eluted using 5 mL of hexane: diethyl ether (80:20) followed by 5 mL of hexane: diethyl ether (60:40). Extracts were dried in a rotary evaporator and if needed placed in an oven at 100 ºC for 5-10 min remove remaining water before adding 250 µL of the silylation reagent (pyridine/hexamethyl disilazane/trimethylchlorosilane, 9:3:1, v/v/v) to prepare the sample for GC injection. The GC-FID analysis was conducted on an Agilent 7890A GC using a 30 m × 0.25 mm × 0.25 µm DB-5 capillary column (Agilent Technologies) with an injection volume of 1.0 µL and helium as the carrier gas at a flow rate of 1.2 mL min^{-1}. The injector

temperature was held at 280 °C at a split ratio of 5. The GC oven program was held isothermally at 150 °C for 8 min; then ramped at 20 °C min^{-1} to 290 °C and held for 20 min to obtain a total run time of 37.33 min. The FID temperature was 300 °C. The detector gas consisted of hydrogen (flow rate: 30 mL min^{-1}), air (flow rate: 400 mL min^{-1}), and helium make up gas (flow rate: 25 mL min^{-1}). Peak identification was carried out with both analytical standards campesterol, stigmasterol, β-sitosterol (MilliporeSigma) and by comparing against the sample chromatograms provided in the IOC official method and their relative retention times. Quantification was performed using the peak area and concentration of the internal standard.

Statistical analysis

Principal component analysis was performed using Originlab Corporation software version "OriginPro 2016 Sr2." Confidence ellipses and principal component scores were computed by Originlab.

Results and Discussion

Constructing TAG-PCA plots

The first step in applying the original method to be used for avocado oil was to analyze the TAG profile of authentic samples and then plot them using PCA. Fig 1 shows the PCA plot developed using TAGs as variables. The separation of avocado oil from the common adulterants currently used is promising in this method's ability to differentiate pure and adulterated oils. The four common avocado adulterants are in clusters below the x-axis. High oleic safflower and sunflower oils are highly correlated with oleic-containing TAGs and soybean with linoleic-containing TAGs, which is consistent with their fatty acid profiles. The avocado oil cluster is in black, above the x-axis. It was necessary to not only analyze a group of pure avocado oils, but samples that accounted for a variety of variables as it has been documented that the fatty acid

profile, and thereby triacylglycerols, can change depending on harvest time (Slater et al. 1975, Ozdemir and Topuz, 2004), fruit growing region (Tan et al. 2017), and cultivar (Jorge et al. 2015; Fernandes et al. 2018; Yanty et al. 2011).

The avocado oils in this study accounted for harvest times from early to late season from two growing regions: California and Mexico. Two cultivars were included: Hass, the most common cultivar used to make oil, and Carmen. The oil was extracted in two different ways: using the whole fruit, including flesh, skin, and pits; and just the flesh (Green and Wang 2022a). Impact of these variables on purity parameters such as fatty acids and sterols were previously described in Green and Wang 2022a. Inclusion of oils with this breadth of natural variation to form the avocado oil cluster in Figure 7.1 helps to improve method accuracy, as the power and accuracy of this method is related to the diversity and number of available samples.

Using the TAG-PCA plot to test commercial samples purity

One utility of this method is it can be used as a screening tool for avocado oil purity to minimize the number of samples that need both fatty acid profile and sterols analysis. This method can be implemented by plotting an unknown oil onto Figure 7.1 and if it is inside the 95 % confidence ellipse for avocado oil it is considered "pure," if it is just outside the ellipse further testing (fatty acid profile and sterols analysis) is needed to confirm purity. If the sample is close to another one of the adulterant oil clusters on the PCA, then it is likely adulterated with high amounts of that oil.

A set of known, single-origin, and pure avocado oils that were collected from producers around the world and tested using this method. Each sample was from producing regions of either New Zealand, Kenya, or South Africa. Figure 7.2 shows the Kenya and South Africa samples are located within the avocado cluster and thus are considered pure. The sample from

New Zealand is located next to two other pure avocado oils from this study, that are just outside the 95 % confidence ellipse for avocado oil. In this case, the New Zealand sample would need to go through FAP and/or sterols testing to confirm purity. The fatty acids and sterols profile of this sample and the other samples tested, which confirmed their purity are in SI Tables S7.1 and S7.3, respectively. As this method is used, verified pure avocado oils can be added to the avocado oil cluster, bolstering its accuracy, which will be particularly important for new regions and cultivars.

An additional set of 25 avocado oils on the market (either pure or adulterated) were tested using this TAGs method and results confirmed with traditional methods. Figure 7.3 shows these 25 samples on the original Figure 7.1 PCA plot and labeled according to whether they were determined pure, not pure, or maybe pure according to the TAG method. These results were then compared to fatty acids and sterols results, listed in Tables S7.2 and S7.4. Three samples were considered pure (sample 4, Figure 7.3a; sample 11, Figure 7.3b; sample 24, Figure 7.3d) because they were located inside the avocado oil cluster. One sample 23, shown in light grey in Figure 7.3d was just outside the avocado oil cluster and was labeled as “maybe.” This sample was confirmed to be pure upon fatty acid and sterols comparison.

Figure 7.3 shows that in many cases of adulteration, it was clear that a sample was not only far from the avocado oil cluster, but near an adulterant oil cluster. For example, samples 13-17 in Figure 7.3c are all near the high oleic safflower and high oleic sunflower oil clusters. They likely either contain nearly 100 % high oleic safflower oil or, because the high oleic sunflower oils are in between the safflower and avocado oil clusters, these samples could also be adulterated with high oleic sunflower oil and levels of around 75 % (an approximation based on the proximity between the clusters, discussed in Green et al. 2020a). In total, 21 out of the 25 test

samples were considered not pure according to the TAGs method and in all cases the results were corroborated by fatty acids and sterols.

There were no samples tested using this method that were pure according to TAGs but were not pure according to fatty acids and sterols. There were a couple cases shown in Figure 7.2 (New Zealand sample) and Figure 7.3d (sample 23) in which a pure sample can lie just outside the avocado oil cluster. In these ambiguous cases it will still be necessary to utilize traditional methods for purity confirmation. However, the consistency of the results of this method with the traditional methods lend it to be an effective screening tool particularly since this is rapid, simpler, and less wasteful method. The commercial samples tested in this study indicate that current adulteration of avocado oil is often happening at high levels where most commercial samples are far from the avocado oil cluster and easily identified as being adulterated. In the case of low levels of adulteration, particularly with high oleic seed oils, which could go undetected using TAGs, also cannot be detected using traditional methods, which was described in Green et al. 2022b. These risks should continue to be investigated while improving the accuracy of this method with more verified pure avocado oil and its potential adulterants.

Conclusions

This study applied the previously developed method for olive oil to detect adulteration in avocado oil. The TAG-PCA tandem method is as accurate as using both fatty acids and sterols, indicating the promise for it to be used as a simple and effective screening tool for avocado oil adulteration since it uses less time and resources than the traditional methods. Based on the results of this work, this approach is ready to be utilized in the immediate future for the avocado oil industry and could be included in testing methods for certifications to help consumers gain confidence in the authenticity of the products.

Acknowledgments

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Competing Interests

The authors have no competing interests to declare.

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Figures

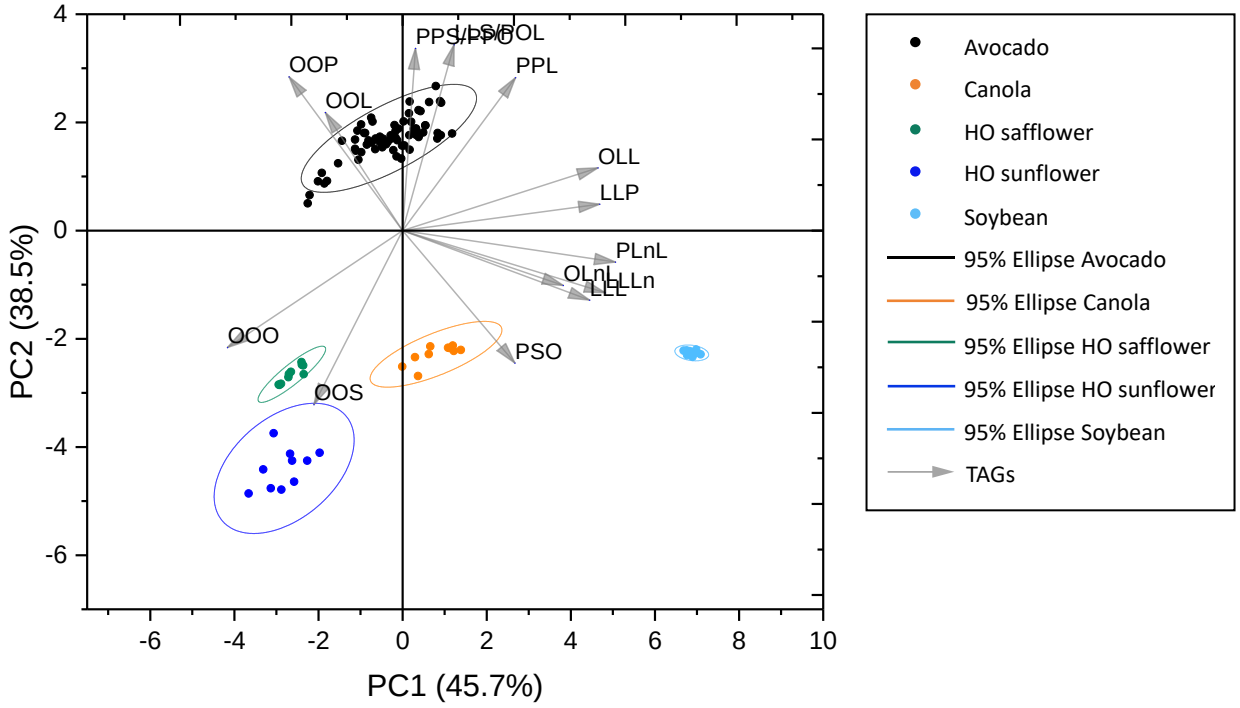


Figure 7.1. Principal component analysis using TAGs as variables showing avocado oil compared to potential adulterant oils. Each oil-type is shown in a cluster of dots, surrounded by a 95 % confidence ellipse with avocado oil in black, canola in orange, high oleic (HO) safflower oil in in blue-green, high oleic (HO) sunflower oil in dark blue, and soybean oil in light blue.

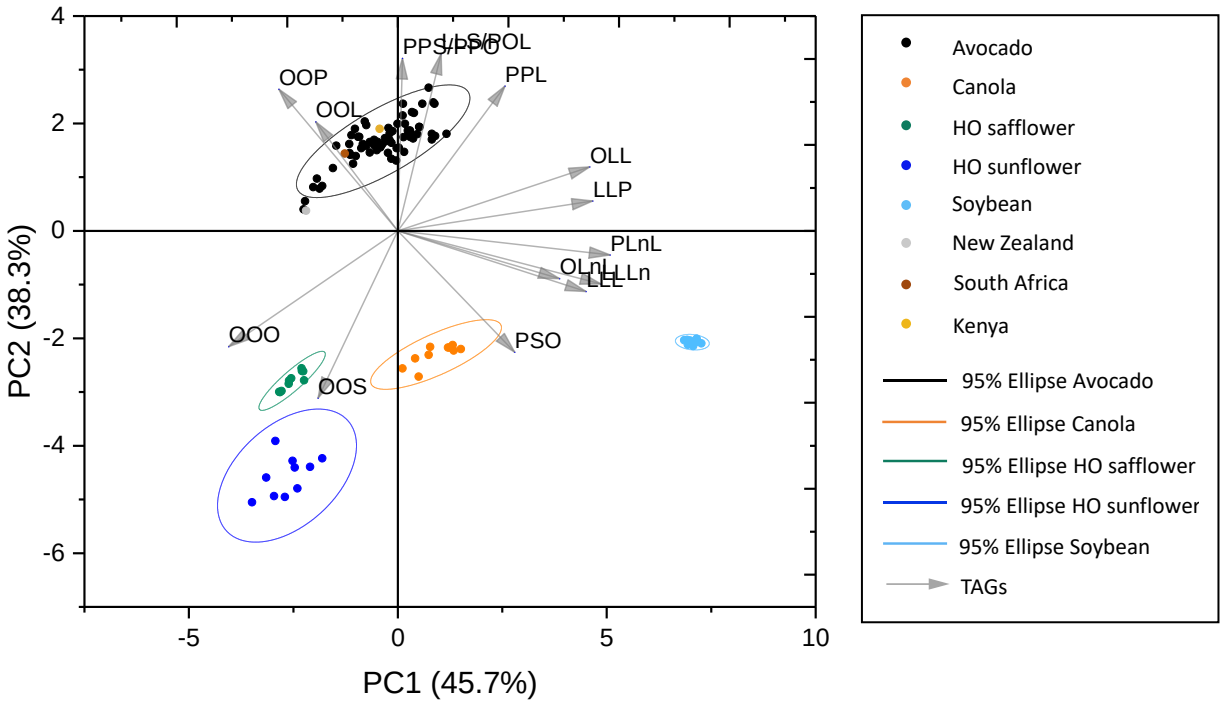
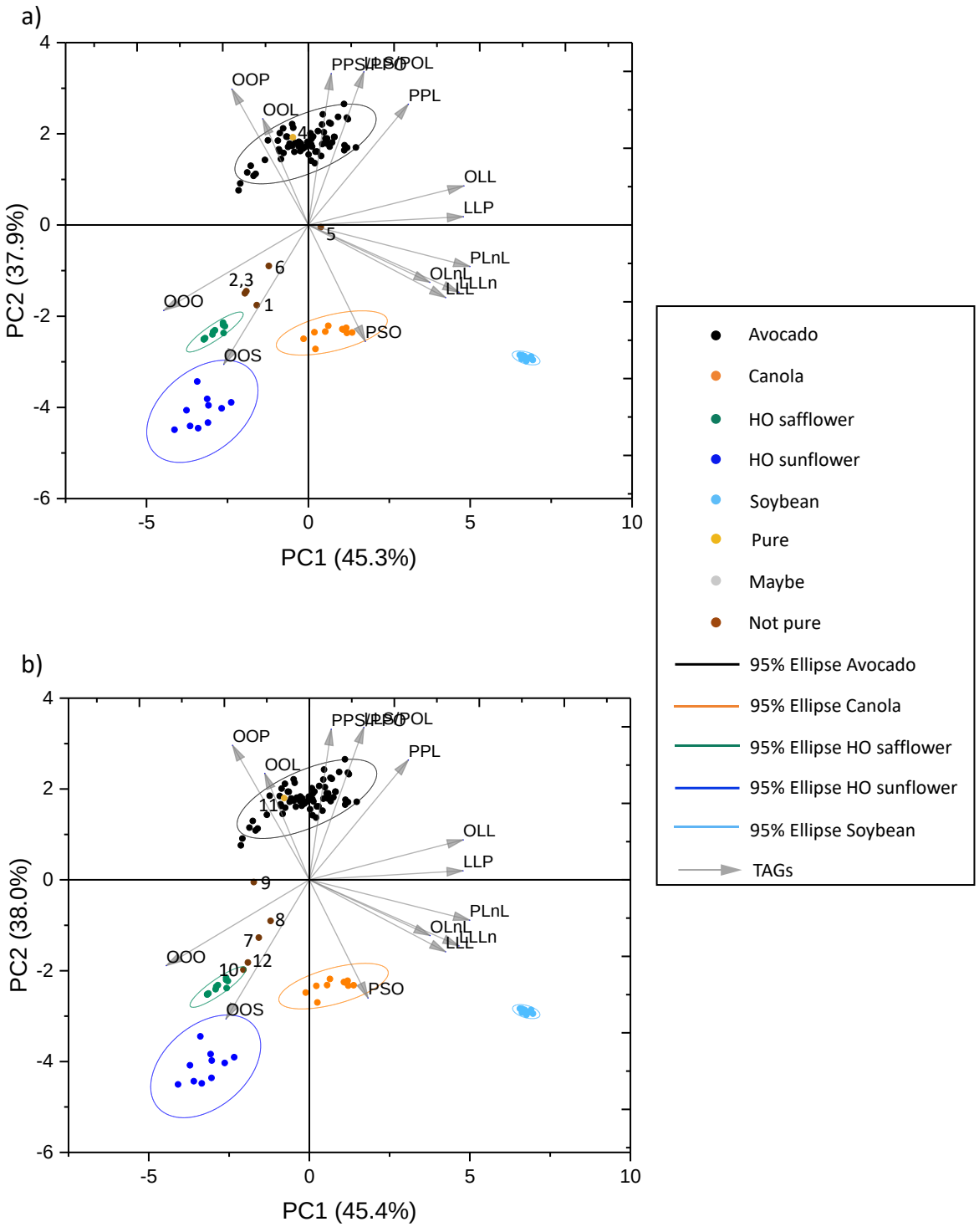


Figure 7.2. Principal component analysis showing the same plot as Figure 7.1 with three pure test-avocado oils also plotted on the PCA from different regions (New Zealand in light grey, South Africa in brown, Kenya in dark yellow). Each oil-type is shown in a cluster of dots, surrounded by a 95 % confidence ellipse with avocado oil in black, canola in orange, high oleic (HO) safflower oil in in blue-green, high oleic (HO) sunflower oil in dark blue, and soybean oil in light blue.


a)
PC2 (37.9%)
PC1 (45.3%)
OOP
OOL
PPS
PPL
OLL
LLP
PLnL
OLnL
LLLn
LLL
OOO
OOS
PSO
1
2,3
4
5
6
b)
PC2 (38.0%)
PC1 (45.4%)
7
8
9
10
11
12
Avocado
Canola
HO safflower
HO sunflower
Soybean
Pure
Maybe
Not pure
95% Ellipse Avocado
95% Ellipse Canola
95% Ellipse HO safflower
95% Ellipse HO sunflower
95% Ellipse Soybean
TAGs

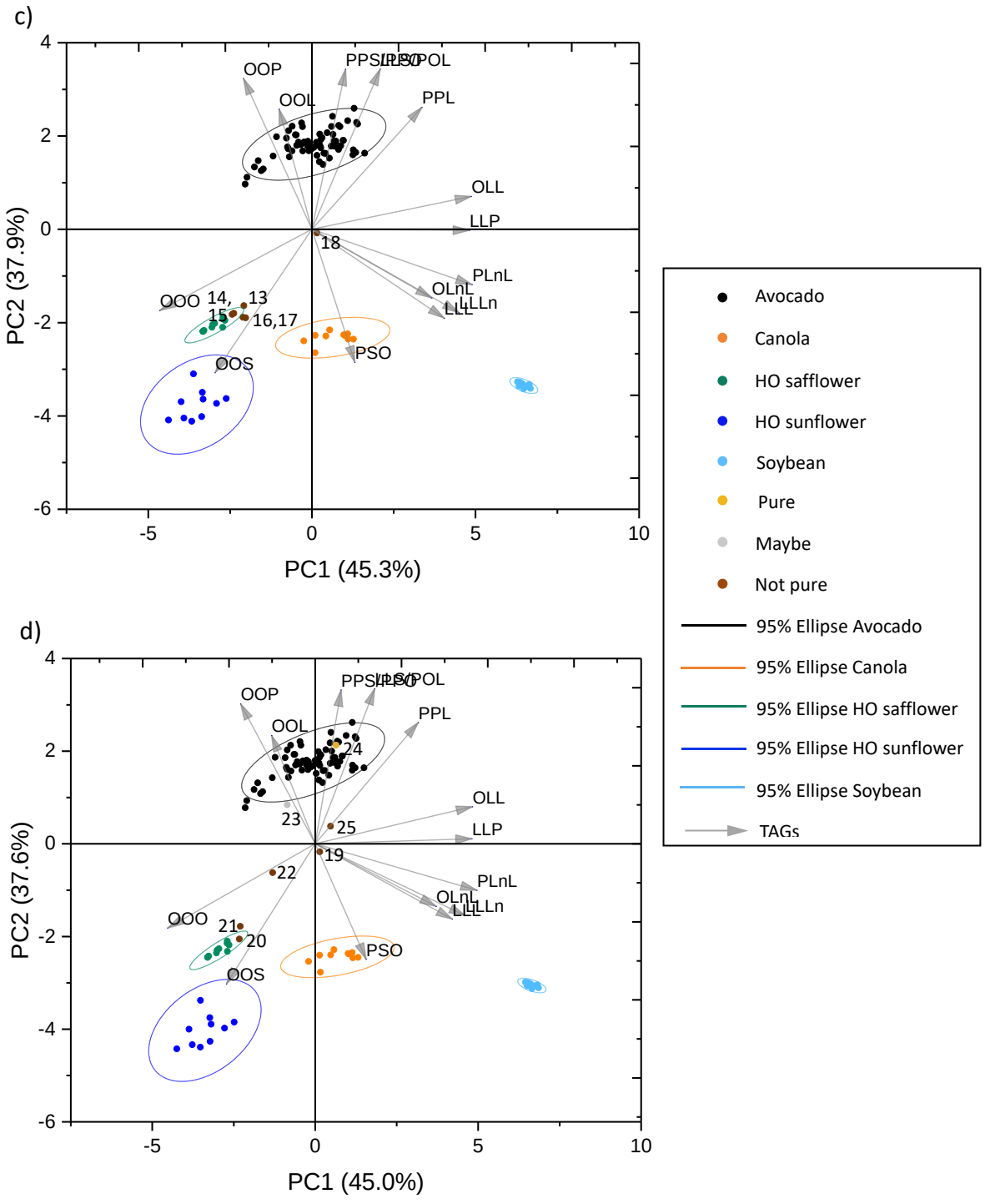


Figure 7.3. Principal component analysis using same clusters as Figure 7.1 with the addition of 25 unknown samples to determine their purity. a) Displays unknowns 1-6; b) Displays unknowns 7-12; c) Displays unknowns 13-18; d) Displays unknowns 19-25 Oils are color-coded according

to whether they are pure according to the TAG method (dark yellow), maybe pure (light grey), or not pure (brown). Each oil-type is shown in a cluster of dots, surrounded by a 95 % confidence ellipse with avocado oil in black, canola in orange, high oleic (HO) safflower oil in in blue-green, high oleic (HO) sunflower oil in dark blue, and soybean oil in light blue.

Supplemental Tables

Table S7.1. Fatty acid values for the pure test samples used in this study presented as average %±SD. Samples in red are outside the current CODEX proposed standards, listed below.

Fatty acid	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0
CODEX	ND-0.3	11.0-26.0	4.0-17.1	0.1-1.3	42.0-75.0	7.8-19.0	0.5-2.1	ND-0.7	ND-0.3	ND-0.5
Kenya	0.05±0	19.77±0	8.73±0	0.66±0	52.18±0	12.18±0	0.88±0	ND	ND	ND
New Zealand	0.03±0	13.85±0	5.63±0	0.46±0	62.76±0.01	10.66±0.01	0.62±0	ND	ND	ND
South Africa	0.04±0	16.96±0.01	6.62±0	0.52±0	57.44±0.01	11.63±0	0.7±0	ND	ND	ND

Table S7.2. Fatty acid values for the unknown test samples used in this study presented as average %±SD. Samples in red are outside current CODEX proposed standards, listed below.

	C14:0 ND-0.3	C16:0 11.0-26.0	C16:1 4.0-17.1	C18:0 0.1-1.3	C18:1 42.0-75.0	C18:2 7.8-19.0	C18:3 0.5-2.1	C20:0 ND-0.7	C20:1 ND-0.3	C22:0 ND-0.5
1	0.04± 0.01	10.88± 0.03	1.62±0	2.79± 0.03	69.88± 0.07	12.98±0	0.65±0	0.42±0.02	0.28± 0.01	0.33± 0.04
2	0.03±0	11.43±0	1.79±0	2.56± 0.02	70.9± 0.01	11.71±0	0.57±0	0.43±0	0.27± 0.02	0.2± 0.01
3	0.03±0	11.32± 0.03	1.77±0	2.56± 0.01	71.14± 0.04	11.58±0	0.57±0	0.44±0.01	0.27± 0.02	0.22± 0.01
4	0.05±0	18.58± 0.02	8.2± 0.02	0.73±0	59.58± 0.03	11.61±0.01	0.75±0	0.15±0	0.19±0	0.06±0
5	0.06±0	13.8± 0.01	4.99±0	1.51± 0.01	58.83± 0.06	17.43±0.02	2.21±0	0.35±0	0.42±0	0.31± 0.08
6	0.06±0	13.09± 0.01	3.6±0	2.24±0	66.82± 0.02	12.66±0	0.56±0	0.36±0	0.25±0	0.26± 0.01
7	0.06±0	11.28± 0.05	2.67±0	2.31± 0.05	69.71± 0.1	12.53±0.02	0.42±0	0.38±0	0.27±0	0.28± 0.02
8	0.05±0	12.33± 0.02	3.06±0	2.11±0	66.94± 0.05	13.94±0.01	0.5±0	0.36±0.02	0.24± 0.01	0.37± 0.01
9	0.04±0	13.57± 0.01	4.41±0	1.42± 0.01	68.46± 0.02	10.58±0	0.7±0	0.29±0.01	0.23±0	0.18±0
10	0.05±0	10.82± 0.01	1.51± 0.02	2.67±0	72.7± 0.01	10.6±0.01	0.5±0	0.42±0	0.28±0	0.36±0
11	0.04±0	17.55± 0.03	8.08± 0.01	0.6±0	60.95± 0.04	11.52±0.01	0.86±0	0.12±0	0.18±0	ND
12	0.03±0	11.07± 0.07	1.58±0	2.67± 0.05	71.08± 0.09	11.82±0.01	0.67±0	0.39±0.02	0.29±0	0.31±0
13	0.03±0	11.23± 0.02	1.9±0.01	2.71± 0.01	71.43± 0.05	11.01±0.01	0.57±0	0.4±0.01	0.27±0	0.36± 0.03
14	0.03±0	11.01±0	1.59±0	2.74± 0.01	73.41± 0.03	9.74±0	0.45±0	0.36±0.01	0.26±0	0.31± 0.02
15	0.04±0	9.79± 0.01	1.39±0	2.66± 0.01	73.85± 0.03	10.59±0	0.55±0	0.37±0	0.29±0	0.39± 0.01
16	0.03±0	10.55± 0.02	1.46±0	2.77± 0.04	71.86± 0.01	11.68±0.01	0.56±0.01	0.38±0	0.27±0	0.34± 0.02
17	0.04±0	9.41± 0.03	2.08±0	2.57± 0.01	73.21± 0.02	11.01±0.01	0.38±0	0.32±0	0.26±0	0.62± 0.01
18	0.05±0	13.9± 0.01	5.12±0	1.41± 0.01	59.48± 0.02	16.91±0	2.16±0	0.28±0	0.39±0	0.22±0
19	0.05±0	13.77±0	5.06±0	1.43±0	59.23± 0.04	17.25±0.03	2.2±0	0.28±0	0.4±0	0.22±0
20	0.02±0	10.79± 0.04	0.7±0	3.12± 0.03	74.32± 0.06	9.28±0.01	0.66±0	0.48±0	0.3±0	0.22±0
21	0.02±0	11.7± 0.01	0.9±0	2.93±0	72.11± 0.01	10.58±0	0.65±0	0.48±0	0.3±0	0.23± 0.01
22	0.04±0	13.41± 0.02	2.91±0	1.96±0	64.61±0	15.63±0	0.61±0	0.32±0	0.23±0	0.2± 0.03
23	0.04±0	16.87± 0.02	5.77±0	1.32± 0.03	61.7± 0.05	12.89±0.01	0.82±0	0.23±0	0.19±0	0.08±0
24	0.05±0	20.95± 0.01	10.21± 0.01	0.75± 0.01	52.31± 0.01	14.28±0	0.95±0	0.13±0	0.19±0	0.12± 0.02
25	0.05±0	14.19± 0.01	4.5±0	1.21± 0.01	55.27± 0.02	23.31±0	0.88±0	0.2±0	0.22±0	0.1±0

Table S7.3. Sterols values for the pure test samples used in this study presented as average %±SD and for total sterols (mg/kg)±SD. Several sterols have two proposed values that are in brackets. For the sake of this study, one of the limits was chosen (in yellow) based off data for the pure samples we have analyzed. Each sterol ends in -sterol, except for delta-7-stigmastenol.

	Chole	Bras-sica	Campe	Stigma	Clero	B-sito	Δ-5-avena	Δ-7-stigma	Δ-7-avena	Total sterols
CODEX	ND-0.5	ND-0.5	4.0-8.3	0.3-2.0	[0.6] [1.0]-2.0	[71.0] [79.0]-93.4	2.0-8.0	ND-[1.0] [3.5]	ND-1.5	[3000] [3500] - 6500 (mg/kg)
Kenya	ND	ND	7.68± 0.05	0.4± 0.01	1.98± 0.06	84.03± 0.29	5.91± 0.29	ND	ND	4649.62± 660
New Zealand	ND	ND	5.72± 0.04	0.36± 0.01	1.81± 0.07	85.45± 0.28	6.66± 0.16	ND	ND	3380.01± 90
South Africa	ND	ND	6.63± 0.07	0.16± 0.22	2±0.07	84.25± 0.47	6.96± 0.54	ND	ND	3018.53± 593

Table S7.4. Sterols for unknown samples as average %±SD and (mg/kg)± SD for total sterols. CODEX proposed standards are listed below. Several sterols have two proposed values that are in brackets. For the sake of this study, one of the limits was chosen (in yellow) based off data for the pure samples we have analyzed. Samples in red were outside proposed standards. Each sterol ends in -sterol, except for delta-7-stigmastenol.

	Chole	Bras-sica	Campe	Stigma	Clero	B-sito	Δ-5-avena	Δ-7-stigma	Δ-7-avena	Total sterols
	ND-0.5	ND-0.5	4.0-8.3	0.3-2.0	[0.6] [1.0]-2.0	[71.0] [79.0]-93.4	2.0-8.0	ND-[1.0] [3.5]	ND-1.5	[3000] [3500]-6500 (mg/kg)
1	ND	ND	7.19± 0.04	3.41± 0.38	0.85± 0.04	75.55±0.71	5.45± 1.36	5.62±0.17	1.94± 0.52	3255±922
2	ND	ND	4.62± 0.04	1.73± 0.03	0.87± 0.04	83.72±0.21	5.05± 0.14	2.63±0.15	1.39± 0.2	2880±190
3	ND	ND	4.81± 0.05	1.81± 0.13	0.94± 0.06	82.49±0.62	4.82± 0.13	3.96±0.19	1.16± 0.32	2864±152
4	ND	ND	6.95± 0.05	0.47± 0.2	1.45± 0.13	85.73±1.14	5.4± 1.12	ND	ND	3716±1220
5	ND	2.07± 0.19	16.1± 0.26	1.94± 0.25	1.16± 0.06	70.52±0.81	5.85± 0.03	1.63±0.27	0.72± 0.28	4195±383
6	ND	ND	7.57±0	2.64± 0.3	1.4±0	78.92±0.01	4.94± 0.1	3.38±0.12	1.15± 0.09	2449±456
7	ND	ND	8.74± 0.16	2.61± 0.15	0.95± 0.02	75.1±1.81	5.49± 0.38	5.79±1.64	1.33± 0.1	2575±626
8	ND	ND	8.34± 0.01	3.74± 0.13	0.87± 0.03	75.96±0.06	4.53± 0.1	4.85±0	1.71± 0.14	2867±275
9	ND	ND	5.9± 0.03	1.7± 0.42	1.45± 0.13	82.29±1.41	5.93± 0.38	1.94±0.18	0.79± 0.33	2956±213
10	ND	ND	7.21± 0.4	3.12± 0.54	0.8± 0.08	76.52±2.97	5.28± 0.89	5.34±1.29	1.72± 0.56	2813±454
11	ND	ND	6.11± 0.05	0.46± 0.27	1.82± 0.13	85.39±0.78	6.21± 0.33	ND	ND	3779±217
12	ND	ND	6.44± 0.01	3.46± 0.27	1.57± 0.51	79.61±0.72	4.41± 0.08	3.36±0.18	1.15± 0.14	2442±170
13	ND	ND	4.92± 0.04	2.57± 0.37	0.88± 0.2	82.42±0.63	3.84± 0.15	4.28±0.08	1.09± 0.34	3128±20
14	ND	ND	8.06± 0.09	3.83± 0.4	1.2± 0.35	75.05±1.21	4.03± 0.2	5.99±0.44	1.83± 0.43	2274±493
15	ND	ND	5.51± 0.01	3.56± 0.46	0.76± 0.14	78.99±0.46	3.36± 0.24	6.34±0.06	1.48± 0.17	2699±140
16	ND	ND	5.29± 0.03	2.93± 0.21	0.72± 0.13	81.98±1.54	4.6± 0.24	3.27±0.61	1.21± 0.32	3256±227
17	ND	ND	8.66± 0.05	6.33± 0.32	0.99± 0.31	66.58±2.82	4.11± 0.27	9.69±2.16	3.65± 0.96	2751±96
18	ND	2.1± 0.04	15.68±0.14	2.05± 0.21	1.06± 0.08	72.77±0.55	5.38± 0.41	0.65±0.12	0.3± 0.06	4715±546
19	ND	2.33± 0.14	16.32±0.48	2.09± 0.28	1.02± 0.33	69.39±1.39	6.31± 0.38	1.7±0.42	0.83± 0.32	3617±152
20	ND	ND	3.48± 0.05	1.74± 0.31	0.96± 0.12	87.44±0.06	5.51± 0.27	0.58±0.15	0.29± 0.03	2620±239
21	ND	ND	3.27± 0.07	1.4± 0.24	1.49± 0.2	87.11±0.21	6.1± 0.22	0.37±0.05	0.24± 0.08	3031±85
22	ND	ND	6.39± 0.04	2.09± 0.15	1.23± 0.22	80.62±0.8	5.84± 0.35	2.87±0.3	0.96± 0.18	2051±107
23	ND	ND	6.05± 0.01	0.6± 0.42	1.65± 0.04	84.29±0.68	7.41± 0.22	ND	ND	3448±496
24	ND	ND	7.84± 0.07	0.76± 0.14	2.09± 0.25	83.89±0.27	5.42± 0.06	ND	ND	4577±317
25	ND	ND	9.86± 0.07	2.49± 0.09	1.15± 0.04	79.48±0.15	6.41± 0.02	0.33±0.07	0.29±0	4298±10