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Research Article

Identification and Characterization of Phytochemicals in Avocado (*Persea americana* Mill, var. Hass) Fruit at Different Maturation and Ripening Stages

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Avocado (*Persea americana* Mill.) is currently among the most demanded fruit crops worldwide due to its nutritional and health benefits. Despite the abundance of reports that have chemically and nutritionally characterized the fruit, information about the characterization of the fruit's phytochemicals at specific maturation and ripening stages is still scarce. This research was aimed to identify and quantify several phytochemicals in 'Hass' avocado fruit at different maturation and ripening stages considering their dry matter values (~20% to over 38%), corresponding to 10.99% and 26.23% oil content, as objective and quantitative parameters of avocado maturation and ripening. Palmitic (1.54–4.46 g/100 g), palmitoleic (0.88–2.60 g/10 0g), and linoleic (0.39–0.93 g/100 g) acids were the dominant fatty acids, showing a decreasing trend during ripening compared to the initiation of maturation (20% DM). β-sitosterol did not change during maturation and ripening, but ergosterol and brassicasterol disappeared at the end of ripening (38.8% DM). β-carotene was maintained at low concentrations (0.04–0.12 mg/100 g) during maturation and ripening, and lutein reached the highest concentration (6.89 mg/100 g) of all the carotenoids at 32.56% DM. α-Tocopherol increased during ripening (up to 0.35 mg/100 g), particularly at 35% DM. Among the phenolic compounds, gallic acid, catechin, chlorogenic acid, and vanillic acid reached their peak concentration (0.32–2.01 mg/100 g) between 33.84% and 36.99% DM. 'Hass' avocados contain significant quantities of bioactive compounds that are of great benefit to human nutrition and health, but their presence and evolution should be well correlated with properly established maturation and ripening stages.

Keywords: bioactive compounds; chromatography; fourier transform infrared spectrometry; mass spectrometry; *Persea americana* Mill.; postharvest

1. Introduction

Avocado (*Persea americana* Mill) cultivation and consumption have increased significantly over the last years, especially in tropical and subtropical regions, with Mexico being the biggest producer, exporter, and consumer [1]. The fruit is characterized by elevated nutritional value with significant amount of nutrients and phytochemicals, which are important promoters of human health [1, 2]. Consequently, world demand for avocados has increased significantly, and it is estimated that the world production of this fruit will increase from the current 6 million tons to 12

million tons by 2030, becoming the most commercialized fruit [3].

Avocado is a climacteric fruit with biochemical and physiological changes during maturation and ripening, including the nutritional and phytochemical composition [4, 5].

Despite several advances in the identification and quantification of avocado phytochemicals, little is known about the contents and changes of several bioactive compounds, especially at well-defined fruit maturation and ripening stages, and much less at unripe stages of the fruit, probably because unripe avocados are inedible. Several authors rely on the ripening or maturity classification of avocado, based on methods such as color charts [6, 7], fruits' texture (firmness) [8], or even image analysis [9], which could offer some practical approach into avocado classification. Nonetheless, these procedures offer responses that are highly variable between avocado varieties, considering the plethora of factors affecting avocado maturity and ripening, and these methods present errors ranging from 10% to 20%, which is an enormous value considering the avocado production [9, 10]. Hence, defining maturity and ripening stages of avocados is critical as these stages have physiological and economic implications in the avocado composition. Indeed, although dry matter and oil accumulation are closely related, the measurement of dry matter (DM) is simpler to conduct, and it is currently recognized as the standard maturity measurement [11]. Particularly for avocado, DM levels close to 19%–22% have been proposed for physiologically mature fruit (called "maturity") [12], while DM of about 24%–28% are characteristics of ripe fruit, which are the consumers' preferred stages [13]. However, a significant amount of avocados, most commonly at the immature and unripe stages, are wasted before and after harvest [1]. Important components, especially phytochemicals, of the wasted fruit, can be used as ingredients in the food, pharmaceutical, and other industries. Therefore, detailed characterization of fruit composition is still needed over a wide range of fruit developmental stages, not only the edible ones [14].

Considering avocado phytochemicals, increases in avocado fatty acids such as palmitic, linoleic, and linolenic acids in 'Hass' avocados during 12 [4] and 21 days [15] of postharvest ripening have been reported, but the absence of oleic acid after 8 days of postharvest ripening [16] could indicate either no clear behavior of the content of fatty acids or differences due to the different methods used, not only the analytical methods but also those for determining fruit ripening stages and extraction methods. Changes in fatty acids have not been commonly correlated with well-defined maturation and ripening stages using proper indices such as fruit DM content.

Similarly to fatty acids, contradictions in the behavior of tocopherols suggest the need for studying their content in avocados, considering their DM values. For instance, while a 23% decrease in α-tocopherol in avocados stored for two weeks at 20°C was observed [17], no consistency was found for the same compound in avocados stored for two weeks at 20°C [18]. In contrast, Villa-Rodriguez et al. [5] showed α-tocopherol and γ-tocopherol increases during 12 day of postharvest ripening, but δ-tocopherol was only detected in fruit after 8 days of postharvest ripening. Ashton et al. [19] demonstrated that total carotenoids in 'Hass' avocado decreased from 2.5–5.5 μg/g to 0.5–2.5 μg/g after 13 days of postharvest ripening, with lutein being the most abundant in fruit with green and yellow flesh, while chlorophyll content increased during the first 9 days but tended to decrease afterward. However, Donetti and Terry [20] reported no significant differences in total carotenoids of 'Hass' avocado during 7 days of postharvest shelf life. Cervantes-Paz et al. [18] reported a decrease in neoxanthin, violaxanthin, lutein, luteoxanthin, and *cis*-violaxanthin in avocado fruit after 16 days of postharvest ripening, as well as chlorophyll *a* and *b*.

Therefore, there are still limited and contradictory information on the content and the evolution of important avocado fruit phytochemicals, especially those of health importance, during very well-defined, wide-range stages of fruit maturation and ripening. In order to evaluate the hypothesis that avocado fruit is rich in phytochemicals, but their presence and quantities depend on well-defined fruit maturation and ripening stages, which need to be considered for the health benefits of the consumer, as well as for proper handling of the fruit and its use in different industries, the present study reports the use of several quantitative techniques to identify, quantify, and investigate the evolution of fatty acids, chlorophylls, carotenoids, tocopherols, and phenolic compounds in 20 well-defined fruit stages from maturation (19.97% DM, 11.00% lipids) to senescence (up to 38.84% DM, 26.23% lipids) of 'Hass' avocados.

2. Materials and Methods

2.1. Fruit Source and Initial Handling. Physiologically mature avocado fruit (*P. americana*, cv. Hass) were harvested at 19.97% DM and 11.00% of lipids from a commercial orchard in Uruapan, Michoacán, Mexico. Homogenous, defect-free fruits were selected on arrival at the laboratory 4 h after harvest. The fruit were washed, weighed, and labeled, and a sample of 15 fruit was randomly selected to evaluate the initial conditions of the received fruit; the rest were stored at 28 ± 2°C and 80 ± 5% relative humidity. Starting the second day of storage, 3 to 5 of the least ripe fruits were selected for daily analysis carried out on individual fruits and repeated for the following 15 consecutive days.

2.2. Analysis of Physicochemical Attributes. Tristimulus Color. Fruit peel color was determined longitudinally at six points of each avocado fruit with a CM 2002 colorimeter (Minolta Co. Ltd, Osaka, Japan) using the CIEL*a*b* scale calibrated with the white pattern during each sampling time. The objective color determination included the parameters a^* (indicating changes from green, $-a^*$, to red, $+a^*$, color), b^* (indicating yellow, $+b^*$, to blue, $-b^*$, color), L^* (indicating lightness or brightness, 0 = black to 100 = white), color saturation, C^* (indicating vividness), and hue, h° (indicating color angle).

Dry matter content (DM). DM was determined in the fruit pulp (flesh) [16]. Avocado fruit were quartered longitudinally, skin and seed removed, and slices (1-2 mm) of

pulp tissue were taken from each quarter, put in Petri dishes, weighed, and dried in a microwave oven (Daewoo model KOR-142HMB, 800 W, Daewoo, Seoul, South Korea) to a constant weight. Microwave was used as a standard technique for DM calculation since the most commonly used procedure the avocado industry. In addition, microwaving allows rapid dehumidification and can be easily monitored [21, 22].

2.3. Fourier Transform Infrared Spectroscopy (FTIR) Analysis. FTIR analysis was performed using a Cary 630 FTIR equipment (Agilent Technologies, Inc., Santa Clara, CA, USA). Samples of 0.5 g of fresh pulp were placed on a diamond crystal of the FTIR system, and the infrared spectra were recorded in the 4000–650 cm^{-1} range at 4 scans per sample with a resolution of 4 cm^{-1}. Supporting Table S1 shows the identified functional groups and their reported absorptions that were used to analyze data obtained from different maturation and ripening stages.

2.4. Identification and Quantification of Chemical Composition

2.4.1. Freezing and Freeze-Drying Preparation of Samples. Immediately after separating samples for the analysis in the fresh pulp tissue, the rest of the pulp was frozen in liquid nitrogen, kept at −80°C and freeze-dried in a Labconco model 779 freeze drier (Labconco Co., Kansas City, USA) at −40°C and 0.03 mBar for 72–96 h.

2.4.2. Oil Content and Fatty Acids Composition. Samples of freeze-dried powder (1 g) of the 20 fruit stages were extracted with hexane (150 mL) in a FOSS Soxhlet 2055 semi-automatic system. The samples were refluxed for 5 h at 70°C, then the hexane was evaporated in an oven at 65°C. The oil content was determined gravimetrically, placed into amber glass vials, flushed with nitrogen, and stored at −20°C until analysis of fatty acids composition. Extracted oil samples (50 mg) were placed in 15 mL polypropylene tubes and saponified with 1 mL of 0.5 N KOH in ethanol. The tubes were heated at 90°C for 10 min, followed by the addition of 1 mL of BF_3 and heated at 90°C for 5 min. Hexane (1 mL) was added, the samples were reheated at 90°C for 3 min, and 1.5 mL of hexane was added. Finally, the samples were centrifuged (9000 × g for 20 min at 4°C), and the organic phase recovered and evaporated in a rotary evaporator (350 mbar and 40°C). The residue was reconstituted in HPLC grade hexane, filtered, and automatically injected (1 μL) into a 7820A gas chromatograph, GC (Agilent Technologies, USA) in the splitless mode. The fatty acid methyl esters were separated in an HP 88 column (60 m × 0.25 mm, 0.20 μm I.D.). The oven temperature program started at 120°C, maintained for 1 min, and then increased to 175°C (10°C/min), maintained for 10 min and increased to 210°C (5°C/min), and kept for 10 min. The final temperature was 230°C (5°C/min) and the total running time was 47 min. Nitrogen was used as the carrier gas with a constant flow of 0.5 mL/min. The fatty acid methyl esters were monitored with a flame ionization detector (FID) set at 250°C. Commercial standards of high purity (> 95%) were used for identification and quantification, including methyl myristate, methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate, methyl laurate, and methyl arachidonate. Quantification was accomplished using calibration curves of the HPLC-grade commercial standards made with dilutions of each compound independently, and results were expressed as mg/g of dried pulp.

The identified fatty acids were used to calculate the amount of total saturated fatty acids (SFAs) as the sum of lauric (C12:0), myristic (C14:0), palmitic (C16:0), and stearic acids (C20:0); total monounsaturated fatty acids (MUFAs) were calculated as the sum of palmitoleic (C16:1) and oleic (C18:1) acids; and total polyunsaturated fatty acids (PUFAs) were calculated as the sum of linoleic (C18:2) and linoleic (C18:3) acids. The values for these calculations (SFAs, MUFAs, and PUFAs) were expressed in g/100 g.

2.4.3. Quantification of Chlorophylls, Carotenoids, Tocopherols, and Phenolic Compounds by HPLC. For chlorophylls, carotenoids, and tocopherols, freeze-dried samples (0.5 g) of the 20 fruit stages, prepared as described in Section 2.4.1., were shaken in 10 mL of absolute methanol at 200 rpm for 1 h in the presence of 0.2 g of calcium carbonate, sonicated for 5 min, and ground in a homogenizer (Ika Works Inc., Wilmington, NC, USA). The homogenates were centrifuged at 8000 × g for 5 min at 25°C, and the supernatants recovered. The pellets were washed with 5 mL of a mixture of acetone:hexane (1:1 v/v), and 100 μL of 0.1% BHT were added. Afterward, the mixture was vortexed for 30 s, centrifuged at 8000 × g for 5 min at 25°C, and the supernatant recovered and added to the first methanol extract. Extracts were poured into separatory funnels, and 20 mL of sodium sulfate were added, stirred for 30 s, and 50 mL of distilled water was added and left to settle for 1 h. After that, 30 mL of acetone:hexane mixture (1:1 v/v) was added to recover all the pigments. The water was purged, and the acetone:hexane mixture was removed using rotary evaporation with vacuum at 40°C. The recovered pigments were suspended in HPLC grade acetone, filtered with a 0.2 μm syringe filter, and stored protected from light at −20°C until subsequent HPLC analysis.

The HPLC analysis of individual carotenoids, chlorophylls, and tocopherols was conducted based on the method reported by Cervantes-Paz et al. [16], with some modifications. An HP 1100 series HPLC (Hewlett Packard, Palo Alto, CA, USA) was used, equipped with a diode array detector (DAD) for the detection of chlorophylls and carotenoids, and simultaneously a fluorescence detector (FLD) for the analysis of tocopherols. The stationary phase consisted of a C30 carotenoid reversed-phase column (4.6 × 150 mm, 3 μm) (YMC Inc., Milford, MA, USA). The mobile phase consisted of methanol as solvent A, water as solvent B, and methyl tert-butyl ether (MTBE) as solvent C. In the elution gradient of methanol and MTBE, water at 4% was used as isocratic flow. The gradient program of methanol and MTBE was 94.5% at min 0, decreasing at min

31% to 68%; 54% at min 50%; 54% at min 50-52; increasing to 94.5% at min 52–60 to return to initial conditions, and finally, 5 min to post-run. The flow rate was 0.75 mL/min, and the injection volume was 10 μL. The carotenoids and chlorophyll *a* and *b* were monitored using the DAD detector at 441 at 665 and 452 nm, respectively, and UV-VIS spectra for all peaks were recorded from 200 to 700 nm. The tocopherols detected with the FLD were monitored at 295 nm of excitation ($\lambda_{EX} = 295$ nm) and 320 nm of emission ($\lambda_{EM} = 320$ nm). High purity (> 95%) commercial standards of xanthophyll, violaxanthin, neoxantin, β-cryptoxanthin, lycopene, β-carotene, chlorophyll a, chlorophyll b, α-tocopherol, δ-tocopherol, and γ-tocopherol were used for identification and quantification. For the quantification of each compound, calibration curves were constructed with dilutions, and results were expressed as mg/g DM.

For phenolic compounds, samples of 0.10 g of freeze-dried avocado powder of the 20 fruit stages were mixed with 3 mL of 80% HPLC grade methanol and sonicated for 60 min, then centrifuged for 15 min at 8000 × g and 10°C, and the supernatant was recovered by decanting. The samples were then filtered through nylon membranes of 0.45 μm pore size (Millipore Corp., Bedford, MA, USA), and analyzed by HPLC. The same HP 1100 series HPLC equipped with a diode array detector (DAD) set at 280 and 320 nm was used, equipped with a 250 × 4.6 mm, 5 μm, RP-18 X-terra RP18 column (Waters). The mobile phase consisted of 1% formic acid (98%) (A) and acetonitrile (2%) (B), at a flow rate of 0.5 mL/min, and 5 μL of samples were injected. The elution gradient was 2% to 100% (B) from 0 to 70 min. Calibration curves for each phenolic standard were prepared for quantification. Chromatograms were monitored at 280 nm, and spectra from UV/VIS (200–700 nm) were recorded. High purity (> 95%) standards of phenolic compounds were used for identification and quantification, and the results were expressed as mg/g DM.

2.4.4. Identification of Carotenoids, Chlorophylls, Tocopherols, and Phenolic Compounds by HPLC-MS. For the HPLC-MS analysis, samples of 6 representative maturation and ripening stages (21.8%, 26.3%, 29.6%, 34.4%, 37.0% and 38.8% DM) were used. Extracts rich in carotenoids, chlorophylls, and tocopherols were injected (10 μL) into the HP 1100 HPLC system equipped with DAD, FLD, and a 6210 time-of-flight (ToF) mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with an atmospheric pressure chemical ionization (APCI) source operating at the positive (APCI+) and negative (APCI-) modes. The HPLC system was equipped with a C30 column (4.6 × 150 mm, 3 μm) (YMC Inc., Milford, MA, USA) operated at 25°C. The mobile phase consisted of water (A), methanol (B), and methyl tert-butyl ether (MTBE) (C). Flow rate was fixed at 0.75 mL/min with the following gradient program: 4% A/94.5% B/1.5% C at 0 min; 4% A/68% B/28% C at 31 min; 4% A/39% B/57% C at 71 min; and 4% A/0% B/96% C, at 85 min. Carotenoids, chlorophylls, and tocopherols were identified by comparing their retention times, UV−VIS, and mass spectra to those of commercial HPLC-grade standards. UV−VIS spectra for carotenoids and chlorophylls were recorded at 200–600 nm. Tocopherols detection was set at 295 nm (excitation) and 320 nm (emission). Mass spectra were obtained in the range of 100–1200 m/z.

Extracts rich in phenolic compounds from fruit samples of the same 6 ripening stages were injected (20 μL) into the HPLC system. The HPLC system was equipped with an Xterra RP18 column (4.6 × 250 mm × 5 μm) kept at 25°C. The mobile phase was 1% formic acid (A) and acetonitrile (B), and the elution gradient was 2%–100% (B) in 40 min at a flow rate of 0.5 mL/min at 25°C. The TOF mass spectrometer was equipped with an electrospray ionization (ESI) source operating at the positive (ESI+) and negative (ESI−) ionization modes and MassHunter manager software (Version A.02.01). Phenolic compounds were identified by comparing their retention times and UV–VIS data with those obtained with reference standards using a mass spectra range of m/z 50–800.

For the mass spectrometer, high-purity nitrogen (99.999%) was used as nebulizing (45 psi) and drying gas (11.0 L/min); gas and vaporizer temperatures were 350°C; the corona, capillary, and fragmentary voltages were 4 μA, 4 kV, and 220 V, respectively. The individual identification of all compounds was achieved by comparing their *m/z* values of each representative peak from their chromatograms using the High-Quality Mass Spectral Database MassBank v. 2022.12 (https://massbank.eu/MassBank), the Dictionary of Natural products (DNP) (http://dnp.chemnetbase.com), and the National Institute of Standards and Technology (NIST) Mass Spectral Library (https://chemdata.nist.gov/).

2.5. Statistical Analysis. Data was presented as the mean ± S.E. of at least three independent experiments in triplicate. All data were normalized to their weight and were expressed as mg per 100 g of dry weight (DW). After normality tests using the Shapiro-Wilk's test, an analysis of variance (ANOVA) was conducted, followed by *post-hoc* Tukey-Kramer's test, where statistical significance was established at $p < 0.05$. Pearson correlations were also conducted. The statistical analysis was conducted in JMP v.17.2.0 (SAS Inc., Cary, IN, USA).

3. Results and Discussion

3.1. Color and Oil Content in Avocado Fruit During Maturation and Ripening. DM of the avocados increased after harvest from 19.97% to 38.84%, corresponding to 11.00% and 26.23% of oil (Figure 1(a)), respectively, indicating that the fruit used in the study are an adequate representative from the initial stage of maturation to over-ripening.

External fruit color intensity (h°) decreased because of increased DM content (Figure 1(b)). Fruit peel color luminosity (L^*) decreased with the accumulation of DM in the fruit, from 22.59 to 5.88 in fruit with DM values from 19.97% to 38.84%, although some of the reductions were not significant (e.g., from 27.74% to 33.63% DM; 34.42% to 38.84%,

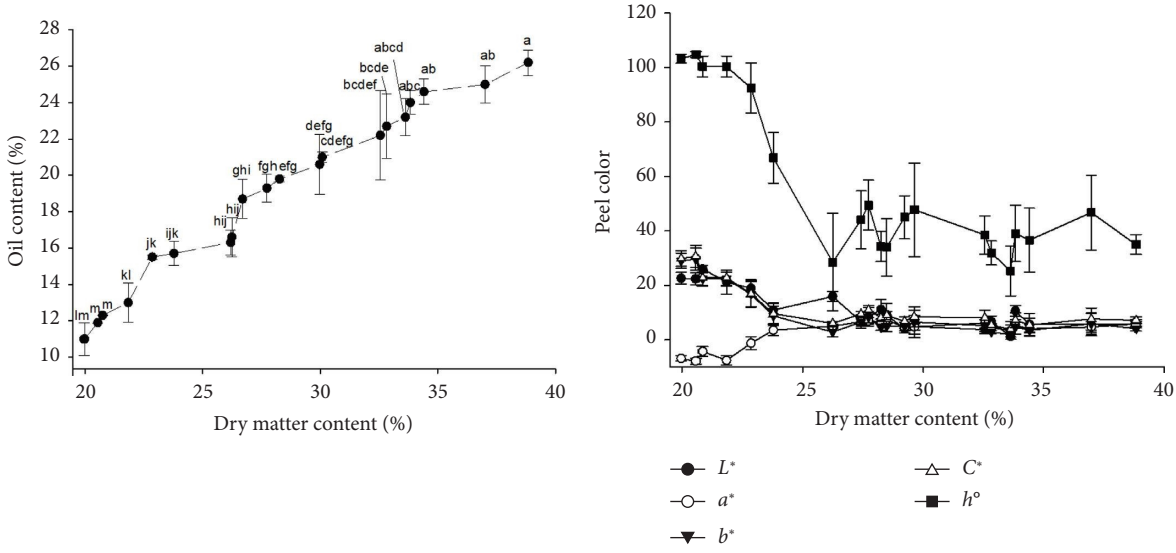

Figure 1: Changes in oil content (%) (a) and peel color (b) in 'Hass' avocado fruit at different maturation and ripening stages, based on dry matter content. Data are expressed as the mean ± S. E. For (a), different letters indicate significant differences ($p < 0.05$) by Tukey-Kramer's test between dry matter stages. For the statistical analysis of (b), please refer to Supporting Table S2.

$p > 0.05$) (Supporting Table S2). The a^* value ranged from −6.82 to 5.85 in the same DM range, indicating a loss of green color and an increase in the dark color during ripening. The lowest b* value was 1.95 in fruit with 33.68% DM, and the highest was 29.64 in fruit with 20.57% DM, indicating a decrease in the yellow color as the fruit matures and ripens. This change in peel color is due to the degradation of chlorophyll but is also influenced by changes in other pigments such as carotenoids, some chlorophyll derivatives (chlorophyllides and pheophytins), and some anthocyanins [2, 23]. These pigments have been reported to have a strong impact on fruit color after harvest [19]. Likewise, the increase in black color during ripening, characteristic of some avocado cultivars such as 'Hass', indicates an increase in anthocyanin-type flavonoids [19, 23]. The color change in fruit peel and pigments variations are influenced by several factors, including the genetic variety of the avocado [1]. It can be noted in Figure 1(b) that the most relevant changes of the color coordinates (L*, a*, b*, C* and h°) of 'Hass' avocado fruit occur between 19.97% and 25.00% DW and remain constant afterward.

Figure 2 shows the content of some fatty acids in the 'Hass' avocado fruit pulp. Eight fatty acids were found to be dominant in the esterified oil of avocado pulp, including oleic, palmitic, palmitoleic, linoleic, linolenic, stearic, myristic, and lauric, in order of abundance, respectively. Other fatty acids detected in smaller quantities included arachidonic, lauric, behenic, *cis*-11, 14-eicosanoic, *cis*-10-heptadecanoic, γ-linolenic and heneicosanoic acids. Statistical differences were noted in the content of fatty acids identified (Figure 2 and Supporting Table S3). The primary fatty acids were: oleic acid, with values ranging from 25.92 g/100 g with 21.83% DM to 6.68 g/100 g in fruit with 33.63% DM; followed by palmitic acid with values ranging from 4.46 g/100 g in fruit with 21.83% DM to 1.54 g/100 g in fruit with 36.99% DM; palmitoleic acid with values ranging from 2.60 g/100 g in fruit with 20.04% DM to 0.84 g/100 g in fruit with 33.63% DM; and γ-linoleic acid with values ranging from 0.93 g/100 g in fruit with 27.74% DM to 0.33 g/100 g in fruit with

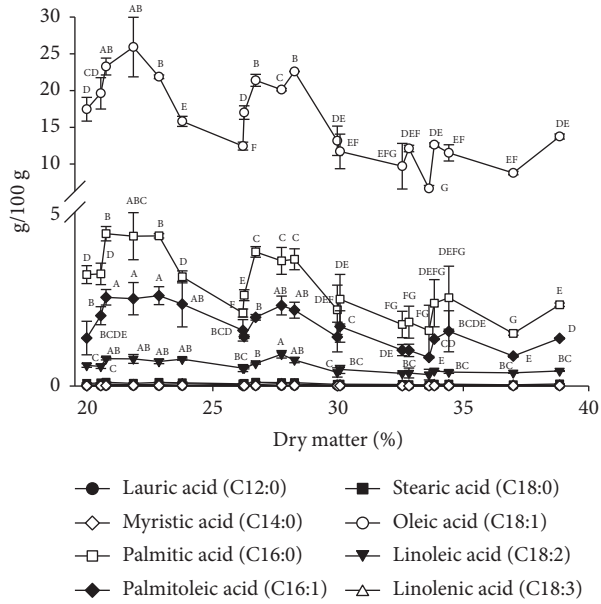

Figure 2: Fatty acid content for each analyzed dry matter (%) value in 'Hass' avocado fruit at different maturation and ripening stages. Data are expressed as the mean ± S. E. Different letters indicate significant differences ($p < 0.05$), for each fatty acid, between dry matter stages, by Tukey-Kramer's test. Numerical data and statistical analyses can also be found in Supporting Table S3. A representative gas chromatogram of each DM can be found in Supporting Figure S1.

33.63% DM (Supporting Table S3). The concentrations of these fatty acids were higher in the early stages of maturation and ripening and decreased as ripening progressed. Lauric acid ranged between 0.004 to 0.016 g/100 g, linolenic acid from 0.020 to 0.069 g/100 g, and stearic acid from 0.032 to 0.108 g/100 g. In addition to showing the lowest concentrations, these three fatty acids remained stable during fruit maturation and ripening. This fatty acids profile is similar to what was reported by Páramos et al. [24] and Ramos-Aguilar et al. [2] for 'Hass' avocado. A representative GC chromatogram can be found in Supporting Figure S1.

An increase in the oil content was observed as the DM augmented, with 10.99% oil content in fruit with 19.97% DM to 26.23% oil content in fruit with 38.84% DM (Figure 1(a)), agreeing with the reported fatty acids increases along with the maturation and ripening. The identified fatty acids were used to calculate total SFAs, MUFAs, and PUFAs (Supporting Table S4), where all data followed a similar trend: an increase from 19.97% to 20.84% DM, a decrease from 20.84% to 26.25% DM, another increase peak from 26.25% to 28.26% DM, and a gradual decrease up to the end of ripening (38.84%). This is consistent with other reports indicating that oleic acid is the main fatty acid in 'Hass' avocado, followed by palmitic, linoleic, and palmitoleic acids, explaining why MUFAs and SFAs are the most abundant groups of fatty acids [25]. A previous report associated the decrease of PUFAs in the last stage of avocado maturation with higher levels of lysophosphatidylcholine acyltransferase and phospholipid: diacylglycerol acyltransferase, suggesting increased regulation of the glycolysis and fatty acids biosynthesis [26].

As PUFAs and MUFAs are some of the most abundant fatty acids in avocado, they are the most studied chemical families in this fruit and are important contributors to human health benefits reported found in this fruit [27]. A proper SFAs and PUFAs balance, expressed in the PUFAs/SFAs ratio, is essential as a proper amount of PUFAs consumption is needed to regulate endogenous cholesterol synthesis without affecting lipemic alterations potentially induced by excess of SFAs consumption [28]. The calculated PUFAs/SFAs values of this research are very comparable to other reported ratios for 'Hass' avocado pulp [29].

3.2. Identification and Quantification of Phytochemicals in Avocado at Several Maturation and Ripening Stages. Results from the tentative identification of the bioactive compounds using HPLC-MS are shown in Table 1. As observed, 13 compounds, including 4 phytosterols, 2 carotenes, 6 xanthophylls, and one tocopherol, were identified using the APCI source. 'Hass' avocado fruit with 21.8% and 37.0% DM contained the most compounds (11), whereas the fruit with 29.6% DM had the least number (9) of compounds. Among the phytosterols identified, β-sitosterol was identified in all 6 fruit-ripening stages tested. β-carotene was also found in all the 6 maturation and ripening stages, but α-carotene was not found in fruit with 34.4% and 37.0% DM. Except for neoxanthin, zeaxanthin and auroxanthin, all xanthophylls were found in fruits of all the 6 maturation and ripening stages tested. Neoxanthin was detected in the latest ripening stages (37.0% and 38.8% DM), and auroxanthin was present in fruit with 21.8% and 34.4% DM. α-Tocopherol was detected in fruit with 21.8% to 38.80% DM.

Using the ESI ionization mode, 9 compounds were identified, including one hydroxybenzoic acid, 2 hydroxycinnamic acids, one flavone, 2 flavonoids, and 3 anthocyanidins (Table 1). Avocados with 26.3% DM (less ripened fruit) contained all these compounds, but fruit with 29.6% DM had the least number of compounds (7). Among the identified compounds, 2-hydroxycinnamic acid, *trans*-cinnamic acid, kaempferol, and citric acid were detected in fruit at all 6 maturation and ripening stages. The identified flavonoids were not found in fruit at the beginning of the ripening stages (21.8% DM), and procyanidins (B1 and B2) were absent in the studied fruit variety in advanced stages of ripening (with 29.6% and 34.4% DM).

The APCI source has been used to identify lipid-soluble compounds, and it is more suitable than other ionization types for thermally stable polar and non-polar compounds [30]. ESI is an ionization source appropriate for polar organic compounds, and therefore, it has been used to identify water-soluble compounds, preferentially inducing the formation of de-protonated or protonated molecules without fragmentation [31]. These sources can be set to positive and negative ionization modes, forming positively or negatively charged ions, respectively [32]. Although ESI+ is usually preferred because more compounds are expected to be ionized in this mode, ESI- has been proven suitable for improved sensitivity and lower detection limits [33]. APCI is preferred over ESI because less matrix interference is presented since the ionization occurs in the gas phase [34].

Chlorophyll *a* decreased significantly with advanced fruit ripening, reaching zero in fruit with 26.25% DM (Figure 3(a) and Supporting Table S5), and chlorophyll b was very low until the fruit reached 32.83% DM, and zero in more ripe fruit. The results obtained in terms of the decrease in chlorophyll *a* agree with those reported by Ramos-Aguilar et al. [2, 23] for 'Hass' avocado. Carotenoids detected included violaxanthin, lutein, and β-cryptoxanthin, which decreased with advanced fruit ripening (Figure 3(a) and Supporting Table S5). The carotenoid profile found agrees with some of the compounds reported by Ramos-Aguilar et al. [35], and the decrease of these compounds due to advanced ripening has also been reported by Villa-Rodriguez et al. [5]. The three tocopherols (α, δ, and γ) were quantified, but α-tocopherol had the highest concentration and the other two were very low, whereas α-tocopherol has shown an increasing tendency as fruit ripening advances (Figure 3(b) and Supporting Table S5). Cervantes-Paz et al. [16] identified and quantified α- and β-carotene in 'Hass' avocado oil from fruit with 19% to 29% DM, and reported a gradual and progressive decrease of α- and β-carotene, respectively, in fruit with 29% DM, but did not completely disappear, and half of the initial compounds were still found in fruit with 29% DM. The xanthophylls dynamics agree with the reported metabolism of these

Table 1: Tentative identification of compounds in 'Hass' avocado fruit at different maturation and ripening stages, based on dry matter content, using high pressure liquid chromatography coupled with time-of-flight mass spectrometry with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) sources at the positive ($APCI^+$ and ESI^+, respectively) and the negative ($APCI^-$ or ESI^-, respectively) modes.

Ionization mode	Observed RT (min)	λ_{max} (nm)	Dry matter (%)						m/z	Main fragments	Tentative identification
			21.8	26.3	29.6	34.4	37.0	38.80			
APCI											
Phytosterols											
$APCI^-$	14.56	210	*			*	*	*	412.6910	309.2119, 311.1905, 313.1956, 315.2276, 451.2849, 607.4394, 647.4321, 649.4462, 633.4498	Stigmasterol
$APCI^-$	65.09	280	*	*		*	*		396.3392	379.4178, 380.4590, 377.3969, 253.2327, 309.3645	Ergosterol
$APCI^-$	65.24	280	*	*		*	*		398.3548	831.7143, 832.7064, 391.1654, 407.1735, 311.2107, 333.1411, 431.2041	Brassicasterol
$APCI^-$	69.56	210	*	*	*	*	*	*	412.3705	367.3267, 381.3430, 395.3710, 339.2997, 473.2430, 489.2358, 311.2107	β-sitosterol
Carotenoids											
$APCI^+$	4.47	475	*	*	*			*	536.4382	536.5389, 105.9190, 119.0910, 91.0743, 145.1010, 93.0964, 157.1208	α−carotene
$APCI^-$	45.00	452, 478	*	*	*	*	*	*	536.4381	536.5317, 538.0532, 444.4805, 521.5307	β-carotene
Xanthophylls											
$APCI^-$	13.80	419	*	*	*	*	*	*	600.4179	583.4686, 584.4935, 333.2248	Violaxanthin
$APCI^-$	15.72	418					*	*	600.4178	436, 464	Neoxanthin
$APCI^+$	36.30	418				*	*		568.4280	568.3062, 476.3185, 119.0398, 105.0513	Zeaxanthin
$APCI^-$	38.00	419	*	*	*	*	*	*	552.4331	557.4603, 558.4622	β-cryptoxanthin
$APCI^-$	62.35	419	*	*	*	*			600.8700	883.7444, 885.7561, 857.7335, 858.7409, 886.7457, 309.2522	Auroxanthin
$APCI^+$	18.80	419	*	*	*	*	*	*	600.4178	999.0000, 43.0175, 613.0000, 641.0000, 564.0000, 91.0582	Luteoxanthin
Tocopherols											
$APCI^-$	8.30	296	*	*	*	*	*	*	402.3497	402.4948, 137.0109, 177.0715,	δ-Tocopherol

Table 1: Continued.

Ionization mode	Observed RT (min)	λ_{max} (nm)	Dry matter (%)						m/z	Main fragments	Tentative identification
			21.8	26.3	29.6	34.4	37.0	38.80			
ESI											
Hydroxybenzoic acid											
$ESI^-/[M\text{-}H]^-$	9.34	230, 275	*	*		*			170.0215	169.1000, 168.6000, 125.0000	Gallic acid
Hydroxycinnamic acids											
$ESI^-/[M\text{-}H]^-$	32.90	235, 276, 330	*	*	*	*	*	*	164.1600	163.1000, 118.9000	2-hydroxycinnamic acid
$ESI^-/[M\text{-}H]^-$	40.70	278	*	*	*	*	*	*	148.1590	95.0486, 103.0538, 105.0445, 79.0534	Trans-cinnamic acid
Flavone											
$ESI^+/[M+H]^+$	46.70	266, 370	*	*	*	*	*	*	286.04773	287.0549, 213.1950	Kaempferol
Flavonoids											
$ESI^-/[M\text{-}H]^-$	1.41	280		*	*	*	*	*	290.07904	109.0300, 123.0430, 125.0220, 137.0240, 151.0390, 203.0640	(+)-catechin
$ESI^-/[M\text{-}H]^-$	5.66	280		*	*	*	*	*	290.2710	289.0716, 109.0298, 123.0455, 245.0828, 203.0721	(−)-Epicatechin
Proanthocyanidins											
$ESI^-/[M\text{-}H]^-$	35.23	280	*	*			*	*	578.14243	289.0698, 407.0768, 577.1315, 125.0239, 425.0846, 451.0971	Procyanidin B1
$ESI^-/[M\text{-}H]^-$	36.83	280	*	*			*	*	578.14243	289.0724, 407.0780, 125.0244, 245.0816	Procyanidin B2

Note: *Indicates the presence of the compound in fruit with a particular maturation/ripening stage.
Abbreviation: RT; Retention time.

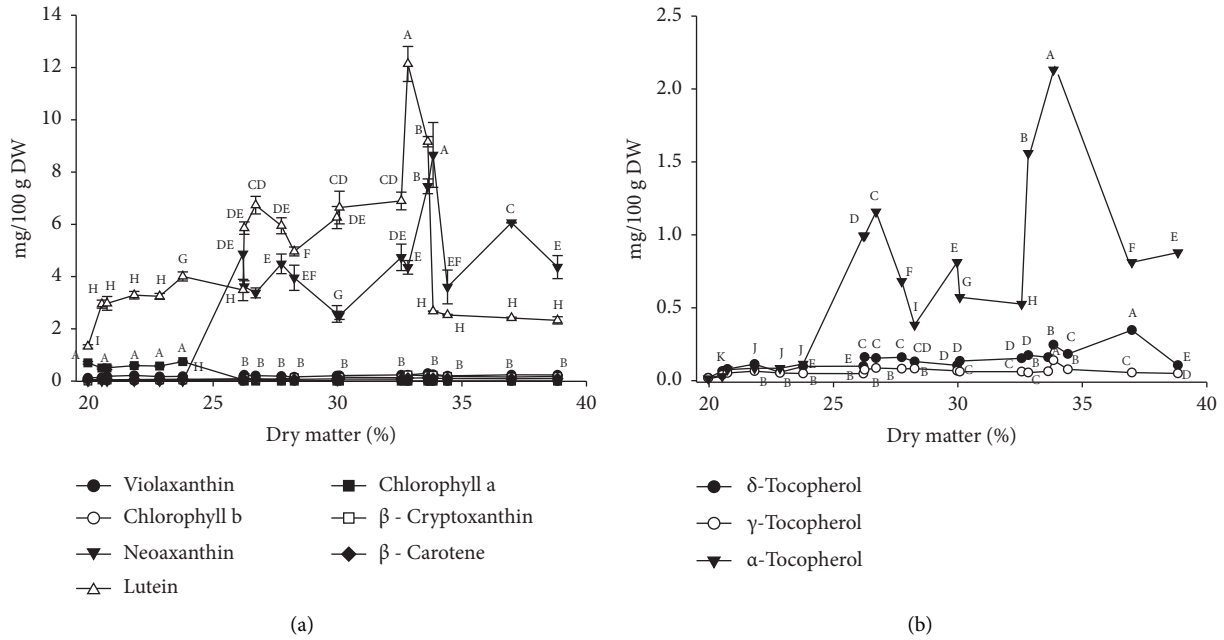

FIGURE 3: Changes in pigments (a) and tocopherols (b) in 'Hass' avocado fruit at different maturation and ripening stages based on dry matter content. Data are expressed as the mean ± S. E. Different letters indicate significant differences ($p < 0.05$), for each pigment, between dry matter stages, by Tukey-Kramer's test. Detailed statistical analyses and numerical data can be found in Supporting Table S5.

compounds from β-carotene as an indication of the metabolic activity of carotenoids [36], since violaxanthin was found in fruit of all the 6 analyzed ripening stages by HPLC-MS (Table 1), indicating a continuous zeaxanthin production from β-carotene [37], which could explain why it was found in fruit with 34.4% and 37.0% DM (Table 1). A similar explanation could be proposed for neoxanthin, the final enzymatic product from violaxanthin, indicating that in fruit with 37.00% and 38.80% DM, β-carotene catabolism could be at its maximum. δ-Tocopherol was found in avocado fruit after 15-day from harvest but could not be quantified after 3, 7, or 11 days after harvest [5].

Some of the phenolic compounds quantified in the pulp of 'Hass' avocado fruit (Figure 4 and Supporting Table S6) included gallic acid, catechin, chlorogenic acid, syringic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, and epicatechin. Their concentration showed significant differences, with values ranging from 0.0 to 2.01 mg GAE $100\,g^{-1}$ depending on the phenolic compound and the fruit development stage (Figure 4 and Supporting Table S6). Some of the phenolic compounds analyzed that increased with fruit DM accumulation included gallic acid at about 30% DM, catechin at about 27% DM, chlorogenic acid at about 35% DM, *p*-hydroxybenzoic acid at about 25% DM, and vanillic acid at about 33% DM, while syringic acid content decreased as DM increased. Some reports [5] have indicated that the content of phenolic compounds in avocado fruit decreases with ripening. However, none of these studies have correlated these phenolic compounds and their changes with very well-defined maturation and ripening stages. It has been reported that the total phenolic compounds content in avocado fruit decreases as ripening advances, although different cultivars display some differences in the compositional dynamics of individual phenolic compounds [38]. The trend observed from gallic acid and epicatechin agrees with what was reported by Villa-Rodriguez et al. [5], where their concentrations increase during 'Hass' avocado ripening, but gallic acid tends to significantly decrease until it reaches low levels (0.094 mg/100 g) 15 days after harvest. Unfortunately, the values reported by Villa-Rodriguez et al. [5] were evaluated in avocados after certain days from harvest, and no determinations of their DM values were presented. Particularly for epicatechin, no specific trend has been reported on its concentrations in 'Hass' avocados with 31%–34% DM since upward and downward fluctuations have been found (from 2.2 ± 0.8 mg/kg to 32 ± 6 mg/kg dry weight) [14]. The concentration of compounds such as 2-hydroxycinnamic acids reported in this study, such as *p*-coumaric acid and its derivatives (*p*-coumaric acid glycoside and acetyl glucoside), has been found to increase in cold-stored 'Hass' avocado pulp from 25.50% to 31.10% DM [39]. High concentrations of hydroxycinnamic acids, hydroxybenzoic acids, and procyanidins are usually accumulated in avocado pulp, which tend to decrease in 'Hass' avocados with 31%–34% DM [14]. It has been reported that β-sitosterol levels in the late avocado ripening stages can reach up to 35.6 mg/100 g [5]. The presence of β-sitosterol during ripening has been associated with increased activity of genes linked to enzymes from the sterols' biosynthesis, such as farnesyl diphosphate synthase, cycloeucalenol cycloisomerase, squalene synthase, and 24-dehydrocholesterol reductase [40]. Stigmasterol was absent in fruit with 26.3% and 29.6% DM (Table 1), probably due to its low abundance among avocado phytosterols, as indicated by Villa-Rodriguez [5]. Ergosterol has been found

to be one of the most abundant phytosterols in 'Lorena' avocado seed oil, together with 5-α-cholestane and stigmasterol [41], and low ergosterol contents (2.59×10^{-5} mg/100 g) have been quantified in the pulp of ripe Nigerian avocados [42]. Ergosterol is a common phytosterol found in commercial avocado oils, having similar concentrations to those found in linseed and olive oils [43]. Like ergosterol, brassicasterol was not identified in fruit with 29.60% and 38.80% DM (Table 1) but has been identified and quantified in freshly extracted oil from ripe 'Lula' avocado (22×10^{-5} g/100 g) [44].

Our findings related to the higher abundance of compounds in over-ripe avocados (34.4%–38.0% DM) might be associated with increases in the enzymatic activity of phenylalanine ammonia-lyase (PAL) in response to ethylene production after ripening. This enzyme is known to affect chlorogenic acid and epicatechin concentrations in avocadoes [4], as it has been observed in the 'Hass' avocado variety. The identification of compounds using ESI modes (ESI^+ and ESI^-) in this study showed that mature avocadoes (21.8% DM) are rich in several water-soluble phytochemicals, except for selected flavonoids, (+)-catechin and (−)-epicatechin. In contrast, ripe avocadoes (29.6% DM) do not contain gallic acid and the identified procyanidins, although these procyanidins were identified in over-ripe avocados. Elucidation of the exact biosynthetic pathways leading to the increase or decrease in the concentrations of several of the studied metabolites deserve additional studies, not only in 'Hass', but in other avocado varieties.

3.3. FTIR Analysis of Avocado Pulp During Maturation and Ripening. Figure 5 and Supporting Table S2 present the main functional groups from 'Hass' avocado pulp at the 20 different maturation and ripening stages (19.97% to 38.84% DM) using FTIR analysis. The FTIR data consisted in identifying the graphical representation of the light absorption spectrum of the functional groups that correspond to the wavelengths, as well as the increase or decrease in light absorption at the wavenumber of the functional group during fruit maturation and ripening. Up to 15 functional representative peaks indicating the presence of functional groups linked to vibrational modes were identified, which correspond to macromolecules (proteins, lipids, polysaccharides, flavonoids), and several other functional groups (alcohols, esters, amides, aldehydes, ketones, alkanes, and phenols) (Supporting Table S2). The identified peaks were associated with several vibrational modes (extension, flexion, symmetric stretching, asymmetric stretching, and scissoring), but extension (peaks 1–4, 9–11, and 15) was the most common and mainly corresponded to alcohols (peaks 1 and 2: 1016 and 1071.93 cm^{-1}), esters (peaks 3 and 10: 1093.68 and 1737.46 cm^{-1}), and flavonoids (peaks 2 and 15: 1071.93 and 3277.92 cm^{-1}). There was an apparent decrease in the intensity of peaks 1 (secondary alcohols), 2 (primary alcohols and flavonoids), 4 (1151.47 cm^{-1}, polysaccharides), 9 (1636.79 cm^{-1}; amides, aldehydes, and ketones), 10 (1737.46 cm^{-1}; ester, lipids, and aldehydes), 11 (2097.25 cm^{-1}, not identified), 12 (2854.74 cm^{-1}; lipids and aldehydes), 13 (2922.48 cm^{-1}, lipids), 14 (2952.93 cm^{-1}, alkanes), and 15 (3277.92 cm^{-1}; alcohol, phenols, particularly flavonoids) as maturation and ripening advanced (19.97% to 33.63% DM) (Figure 5 and Supporting Table S2).

FTIR is a novel non-destructive alternative analysis technique that does not require complex sample preparation. FTIR analysis has been used in some fruits and vegetables [45] to identify functional groups based on their vibrational modes from a variety of polar and non-polar compounds [46]. So far, reports including FTIR evaluating the phytochemical composition of avocado during postharvest maturation and ripening, have been conducted in oil from fruit at different DM [16], but no evaluations considering phytochemicals' screening have been investigated in avocado fruit pulp.

Decreases in the peaks' intensity as maturity and ripening advances agree with reported compositional changes in avocado during maturation and ripening. For example, alcohols like 1-octen-3-ol, 2-methylpropanol, 2-methylbutanol, 3-methylbutan-1-ol, 2-ethyl-1-hexanol, 2-propanol, and 1-butanol are the most abundant volatile compounds in avocado during ripening, followed by several ketones (3-buten-2-one, 1-phenyl-ethanone, propanone, and 2-butanone), and aldehydes (hexanal, benzaldehyde, nonanal, and pentanal) [47]. Since primary and secondary alcohols absorbance is reduced from maturity to ripening, it could be inferred that branched-chain amino acids are being metabolized, either to produce these alcoholic intermediaries or the *de novo* synthesis of proteins (enzymes) involved in pathways required for the ripening process [48], which may agree with the increased protein (peak 7: 1379.53 cm^{-1}) and amide I (1636.79 cm^{-1}) absorbance from less to more mature and ripe avocados. The decrease in the absorbance of aldehydes (peaks 9, 10, and 12) in our study agree with the decreasing trend observed for some avocado aldehydes (e.g., acrolein and pentanal) during storage because of fatty acid oxidation or changes in amino acid metabolism [49].

The results obtained from FTIR (Figure 5, Supporting Table S2) indicated a change in the wavenumber region of spectra with the advanced maturation and ripening of avocado fruit. The different groups of compounds had slightly higher absorbance during the initial ripening stages, mainly in the wavenumber region of 3277.92 cm^{-1} that correspond to the OH functional group of alcohols and phenols (Supporting Table S2). The wavenumber region of 1151.47 cm^{-1}, which corresponds to the carbon-oxygen-carbon (C-O-C) group of polysaccharides and 1245.92 cm^{-1}, which corresponds to the OH group of cutin and polysaccharides, had a slight decrease. Our FTIR findings indicate similar results to those obtained with GC analysis for lipids profile and HPLC and HPLC-MS analysis for pigments, phenolic compounds, and tocopherols, in which we observed that some phytochemicals, like epicatechin, decreased during avocado fruit ripening, while others like gallic, chlorogenic, vanillic and caffeic acids increased with advanced fruit ripening.

Pearson's correlation coefficients are presented in Figure 6**,** where some of the variables displayed significantly high values (Supporting Table S7). The correlation matrix showed that DM and oil content are closely correlated. The correlation

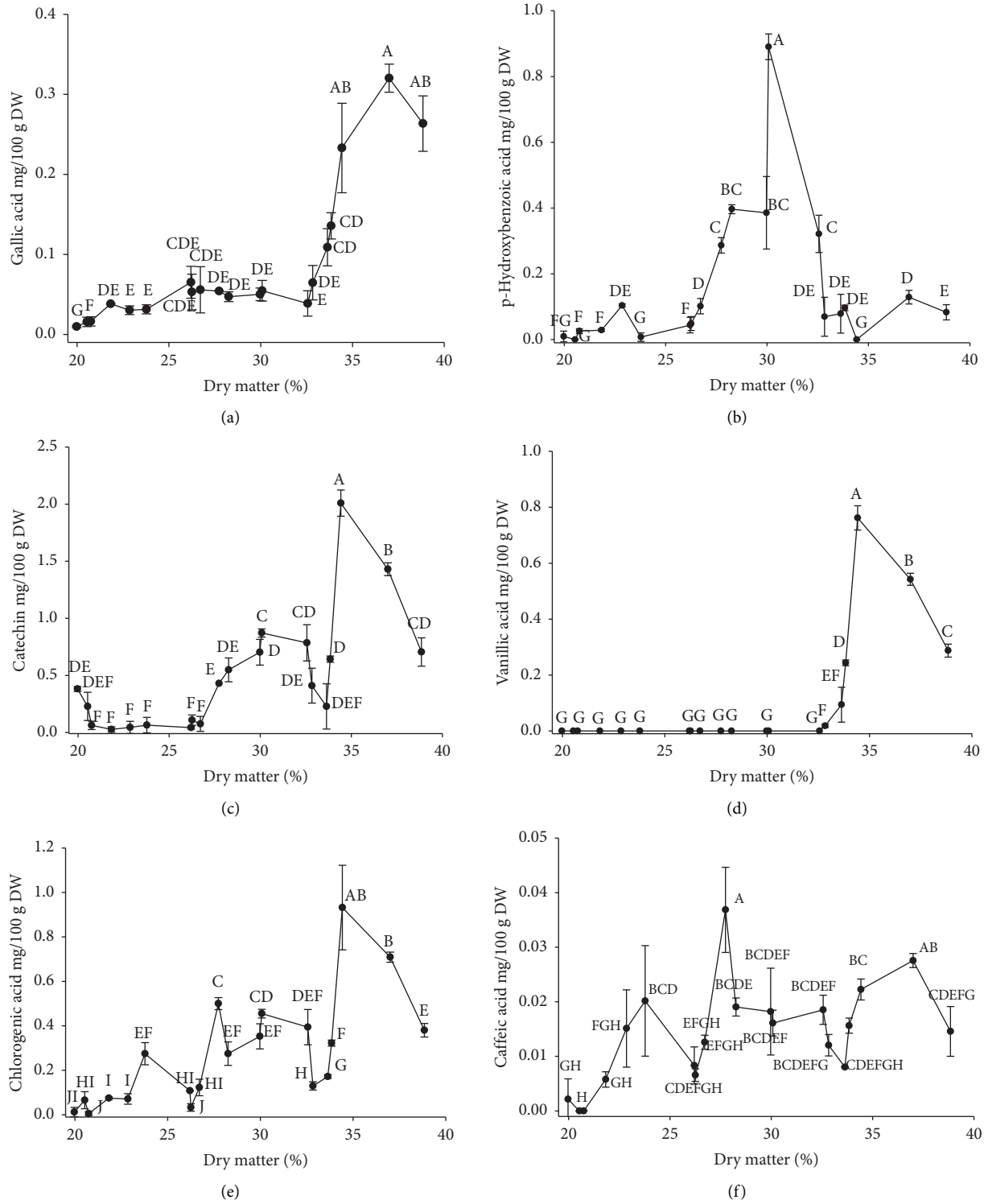

Figure 4: Continued.

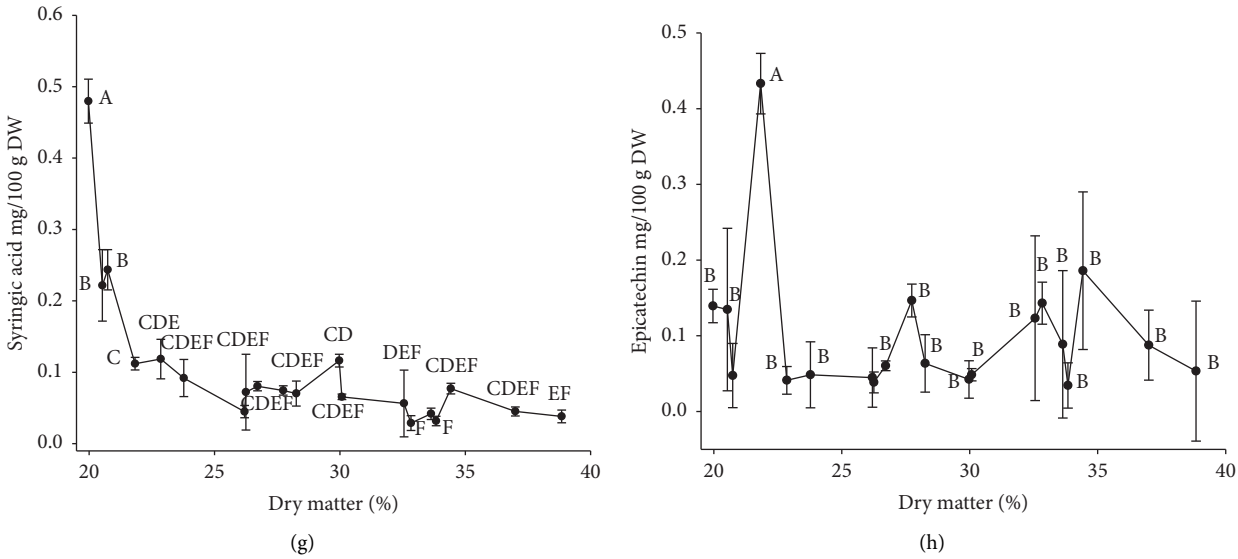

Figure 4: Changes in some phenolic compounds in 'Hass' avocado fruit at different maturation and ripening stages, based on dry matter content. (a) gallic acid; (b) *p*-Hydroxybenzoic acid; (c) (+)-catechin; (d) vanillic acid; (e) chlorogenic acid; (f) caffeic acid; (g) syringic acid; (h) epicatechin. Data are expressed as the mean ± S. E. Different letters indicate significant differences ($p < 0.05$), for each phenolic compound, between dry matter stages, by Tukey-Kramer's test. Numerical data and detailed statistical analysis can be found in Supporting Table S6.

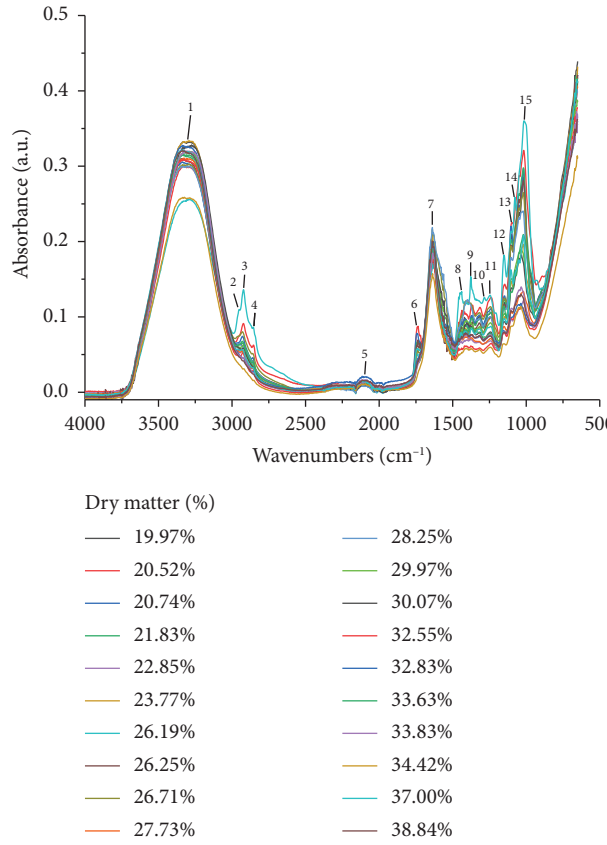

Figure 5: Fourier transform infrared spectroscopy (FTIR) spectra of functional groups in 'Hass' avocado fruit pulp at different maturation and ripening stages based on dry matter content from 19.97% to 38.84%.

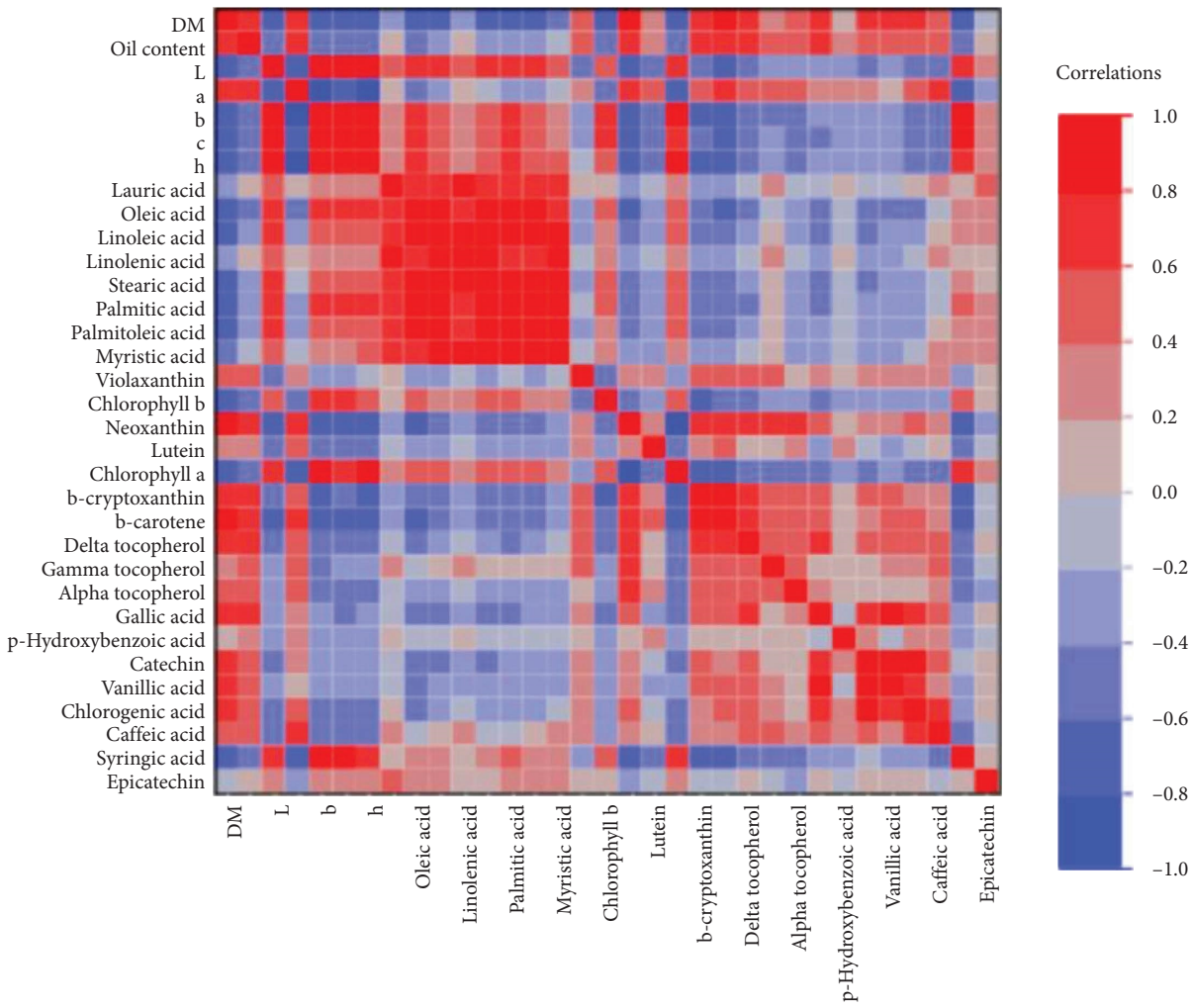

Figure 6: Pearson's correlations between all analyzed variables and dry matter stages. Correlations were performed in JMP v. 17 software. Correlations' values can be found in Supporting Table S7.

matrix demonstrated that the estimated sample correlation coefficient was high (r: 0.95) and was highly significant (p: 0.0001), thus indicating a strong association between the DM and the oil content in 'Hass' avocado fruit. However, additional studies are needed in other avocado varieties and conditions to fully demonstrate this relationship.

4. Conclusion

The results of this study demonstrate that selected phytochemicals from 'Hass' avocado fruit exhibit variations during specific maturation and ripening stages, which can be accurately defined using the fruits' DM and oil contents. Previous reports on identifying and quantifying phytochemicals in avocados lacked correlations with well-defined maturation and ripening stages, thus hindering meaningful comparisons between results. For instance, fatty acids displayed important correlations with the L^* parameter, agreeing with the brightness provided by these components to avocado pulp. In this research, we comprehensively characterized bioactive compounds in avocado fruits, ranging from the initiation of fruit maturation (19.97% DM) to over-ripening (38.84% DM). We offer valuable insights by correlating the maximum concentrations of bioactive compounds with precise fruit stages. This allows for potential applications of these bioactive compounds in various industries, including food, pharmaceutical, and medicinal sectors, throughout all fruit stages, including pre- and post-ripening fruit stages. Moreover, our analysis was conducted at well-defined, wide-ranging fruit developmental stages, facilitating straightforward result comparisons. These findings hold significant importance, as they indicate the appropriate fruit consumption stage to maximize health benefits for consumers and the optimal utilization of the 'Hass' avocado fruit at different developmental stages.

Nomenclature

APCI	Atmospheric pressure chemical ionization
BHT	Butylated hydroxytoluene
DAD	Diode array detector
DM	Dry matter
ESI	Electrospray ionization
FLD	Fluorescence detector

FTIR Fourier transform infrared spectrometry
GAE Gallic acid equivalents
GC Gas chromatography
HPLC High-performance liquid chromatography
MS Mass spectrometry
MTBE Methyl tert-butyl ether
ToF Time-of-flight

Data Availability Statement

Data will be available upon reasonable request.

Conflicts of Interest

The authors declare that there are not conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.
Supporting Table S1. Functional groups, reported absorption locations, and absorption locations from Fourier transform infrared spectroscopy (FTIR) analysis of the identified compounds from 'Hass' avocado fruit at different maturation and ripening stages. Supporting Table S2. Changes in peel color in 'Hass' avocado fruit at different maturation and ripening stages, based on dry matter content. Supporting Table S3. Fatty acids profile in 'Hass' avocado fruit at different maturation and ripening stages, based on dry matter content. Supporting Table S4. Total saturated (SFAs), monounsaturated (MUFAs), polyunsaturated (PUFAs), and PUFAs/SFAs of avocado at different dry matter stages. Supporting Table S5. (A) Pigments and (B) Tocopherol contents of 'Hass' avocado at different maturation and ripening stages, based on dry matter content. Supporting Table S6. Changes in some phenolic compounds in 'Hass' avocado fruit at different maturation and ripening stages, based on dry matter content. (A) gallic acid; (B) *p*-Hydroxybenzoic acid; (C) (+)-catechin; (D) Vanillic acid; (E) Chlorogenic acid; (F) Caffeic acid; (G) Syringic acid; (H) Epicatechin. Supporting Table S7. Pearson's correlation values after correlating the analyzed variables and dry matter (DM) stages. Supporting Figure S1. Gas chromatogram (A) for the presence of fatty acids, 1: lauric, 2: myristic, 3: palmitic, 4: palmitoleic, 5: stearic, 6: oleic, 7: linoleic and 8: linolenic, and their evolution (B) in 'Hass' avocado fruit at different maturation and ripening stages, based on dry matter content. *(Supporting Information)*

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