Secondary Metabolites and Lignin in ‘Hass’ Avocado Fruit Skin during Fruit Development in Three Producing Regions

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Abstract. In plants, secondary metabolites (SMs) have functions of both defense and adaptation to the environment in which they develop. In Mexico, ‘Hass’ avocado is cultivated in different climate types, so during its development, the fruit is exposed to extreme climatic factors, especially temperature and solar radiation. A recent study showed that the thickness and roughness of ‘Hass’ skin increased in the hottest climate. It is unknown how these factors affect the presence of SMs and lignin in the skin. The aim of this research was to quantify the concentration of total phenolic compounds (TPCs), chlorophylls, total carotenoids (TCARs), and lignin in the skin of ‘Hass’ avocado fruit over five developmental stages (S), based on fruit diameter [Olive (20–30 mm), S-I (35–45 mm), S-II (50–60 mm), S-III (60–70 mm)] and Harvest (mesocarp dry matter ≥21.5%), in three producing regions of Mexico: Nayarit (warm subhumid climate, elevation 1151 m), Jalisco (semisubhumid climate, elevation 2180 m), and Michoacán (temperate climate, elevation 1579 m). Both fruit developmental stage and producing region had a significant influence on the concentrations of SMs and lignin in the skin. During fruit development, the skin showed a decrease in the concentration of phenolic compounds (PCs) and an increase in the presence of chlorophylls, carotenoids, and lignin. The skin of fruit produced in regions with a semisubhumid and temperate climate had higher production of lignin and PCs, as well as a lower concentration of chlorophylls.

In addition to the primary metabolism (growth) present in all living beings, plants have a secondary metabolism (protection) that allows production and accumulation of compounds of a diverse chemical nature called SMs, which are distributed differentially among taxonomic groups. According to their biosynthetic pathway, SMs are classified as terpenes, PCs, and nitrogenous compounds (Fang et al., 2011). Generally, SMs are bioactive agents of plants that can interfere with molecular targets in animals and microorganisms; others serve to attract pollinators and animals that disperse their seeds and can also function as antioxidants and ultraviolet radiation protection systems (Verma and Shukla, 2015). SMs play an important role in the adaptation of plants to the environment and in counteracting stress conditions (Ahmed et al., 2014). The defense mechanisms of plants can be structural, such as cell wall thickening or lignification, or biochemical such as the production of PCs, phytoalexins, or enzymes (Anderson et al., 2005). Carotenoids play important roles in plant–environment interaction, including providing essential intermediaries for phytohormone biosynthesis (Esteban et al., 2015).

PC production is affected by the presence of pathogens and other stresses. In avocado (Persea americana Mill.) rootstocks inoculated with Phytophthora cinnamomi, the PC content was higher in asymptomatic plants, indicating that the defense mechanisms were activated (Andrade-Hoyos et al., 2015). In ‘Hass’ avocado fruit, the highest PC concentration was present in the exocarp (skin), probably because of the exposure of this tissue to stress conditions (Tesfay et al., 2010). In smooth-bark Mexican pine (Pinus pseudoostrobus Lindl.) and sacred firs (Abies religiosa L.), the foliar concentration of photosynthetic pigments (chlorophyll A and B, carotenoids) generally decreases in response to stress or disease; on the other hand, environmental factors increase leaf structural chemistry (cellulose and lignin) in these species (Cambrón-Sandoval et al., 2011; Espaía-Boquera et al., 2010).

Factors such as nutrient supply, temperature, light conditions, or carbon dioxide concentration in the atmosphere may influence the concentration of defense-related secondary compounds in plant tissues and, consequently, the distribution of energy expenditure between the primary metabolism and secondary metabolism (Gayler et al., 2008). The contribution of each factor varies between sites and each plant species responds with considerable physiological and genetic plasticity to maintain its dominant status in each habitat.

During plant phenology, the concentration of SMs varies between species, although their quantity and diversity is not universally related to the development of the plant. Early production of these compounds has ecological implications involving defense mechanisms, relationships with microorganisms and role of these compounds as a nitrogen reserve (De-la-Cruz Chacón et al., 2013). The harvest date and the maturity stage of fruit can affect the concentration of TPCs. Golukeu and Ozdemir (2010) found a decrease in PCs in the mesocarp of various avocado cultivars harvested at different dates; a similar phenomenon occurred in Mexican serviceberry [Malacomes denticulata (Kunth) Jones] fruit where PCs decreased during maturation (Herrera-Hernández et al., 2013). In persimmon (Diospyros kaki Thunb.) fruit, the carotenoid concentration decreased during the first stages of development, then increased and reached its maximum value at harvest (Candir et al., 2009).

In Mexico, ‘Hass’ avocado is cultivated in different climates; therefore, the fruit is
exposed to extreme climatic factors, especially temperature and solar radiation. In some markets, the price of ‘Hass’ avocado is lower when it presents pronounced skin roughness (Campos et al., 2011). A recent study showed that the thickness and roughness of ‘Hass’ skin was greater in producing areas with the highest ambient temperatures during fruit development (Salazar-Garcia et al., 2016). PCs are involved in the adaptation of plants to the environment, in particular, their protective role against excessive solar radiation (Alonso-Amelot et al., 2004). It is unknown if environmental conditions affect the presence of SMs and lignin in the skin of ‘Hass’ avocado and if this is related to skin roughness. The aim of this research was to determine the concentration of TPCs, chlorophylls, TCARs, and lignin in the skin of ‘Hass’ avocado fruit during its development in three producing regions of Mexico.

Materials and Methods

Producing regions. Three commercial ‘Hass’ avocado orchards with fertigation were selected in three producing regions (states) with different climate types: “El Parejo” orchard in Tepic, Nayarit, with a warm subhumid climate and elevation of 1151 m; “Paso de Carretas” orchard in Gómez Farías, Jalisco, in a semi-warm, subhumid climate and at 2180 m; “El Parejo” orchard in Uruapan, Michoacán, with a temperate climate and at 1579 m.

Weather data collection. In each orchard, a weather station was installed for the collection of air temperature, precipitation, and solar radiation data at 15-min intervals with automated sensors (mod A753 addWAVE GPRS; Adcon Telemetry, Klosterneuburg, Austria). Using the Microsoft Access 2010 Ver. 14.0 database manager, the meteorological data of the three weather stations were integrated, quantifying the mean monthly temperature, accumulated precipitation, and mean maximum solar radiation.

Fruit sampling and chemical determinations. In each orchard, a group of 30 adult trees [six trees per experimental unit (EU)] were selected. In these trees, fruits corresponding to the main flowering season, which occurred during January and Feb. 2015, were marked. Fruit samplings were performed at different stages (S) of fruit development (Fig. 1), based on their diameter (ø), which were Olive (20–30 mm ø, 8 fruits/EU), S-I (35–45 mm ø, 6 fruits/EU), S-II (50–60 mm ø, 5 fruits/EU), S-III (60–70 mm ø, 4 fruits/EU), and Harvest [when the mesocarp dry matter was ≥21.5%, 4 fruits/EU]. The fruit obtained in each sampling were transported in thermal boxes to the laboratory where they were washed with running tap water, dried with cloth, weighed, and measured. The skin was removed with a vegetable peeler and stored at –80 °C until analysis.

The extraction of TPCs, chlorophyll A, B, and total (C A, C B, and C F); and TCARs were performed on fresh skin with an acetone:water (80:20) extract, according to Rodríguez-Carpena et al. (2011). The TPCs were quantified using the Folin–Ciocalteu method: 500 μL of Folin–Ciocalteu solution (1:10 in deionized water) and 400 μL of sodium carbonate solution (at 7.5%) were added to a 100 μL aliquot of sample. Then, the samples were vortexed and incubated at room temperature for 30 min. Subsequently, the absorbance was measured at a wavelength of 765 nm (Stintzing et al., 2005). The TPC concentration was obtained from a standard curve of gallic acid and expressed in milligrams of gallic acid equivalents (GAEs) per gram of fresh skin (mg GAE/g). Chlorophylls and TCARs were determined with the methodology of Donetti and Terry (2012). Absorbance of the extracts was determined at 470, 647, 663, and 750 nm for turbidity correction. The pigment concentration was calculated using the equations proposed by Lichtenenthaler and Buschmann (2001) and expressed in micrograms of pigment per gram of fresh skin (μg·g⁻¹).

\[
C_A (\mu g\cdot mL^{-1}) = \frac{12.25 A_{663 nm} - 2.79 A_{647 nm}}{} \\
C_B (\mu g\cdot mL^{-1}) = \frac{21.7 A_{647 nm} - 5.10 A_{663 nm}}{} \\
C_T (\mu g\cdot mL^{-1}) = \frac{7.15 A_{663 nm} + 18.91 A_{647 nm}}{} \\
TCARs (\mu g\cdot mL^{-1}) = \frac{(1000 A_{470 nm} - 1.82 C_A - 85.02 C_B)}{214}
\]

The lignin concentration was determined in dry and ground skin subjected to acid hydrolysis with 72% sulfuric acid (Ankom Technology, 2013). A 0.5-g sample was...
placed in a fiber bag, which was introduced into the fiber analyzer for 60 min with a solution of cetyltrimethylammonium bromide to determine the content of acid detergent fiber in the sample. It was rinsed three times with distilled water at 90 °C. The bags were immersed in acetone for 3 min and once dried were placed in the oven at 105 °C for at least 3 h. After acid detergent fiber determination, the bags were submerged in 72% sulfuric acid for 3 h with agitation every 30 min. The bags were then rinsed with hot distilled water until the pH of the water was neutral, then rinsed again in acetone for 3 min and oven dried for 3 h. The lignin percentage in the sample was calculated using the following equation:

\[
\% \text{ lignin} = \frac{\text{bag weight after } H_2SO_4}{\text{bag weight} + \left( \frac{\text{final oven dried blank bag weight} - \text{original blank bag weight}}{\text{sample weight}} \right)} \times 100
\]

Statistical analysis. A 3 × 5 factorial treatment design with three producing regions (Nayarit, Jalisco, and Michoacán) and five developmental stages (Olive, S-I, S-II, S-III, and Harvest) was used. The experimental design was completely randomized with five experimental units, each consisting of six trees. To describe the effect of each factor on the levels of the other, once identified the factors and interactions that had statistical significant effect, an ANOVA and means comparison were performed with the Waller–Duncan test \( P \leq 0.05 \). Pearson’s linear correlation coefficient \( r \) was calculated for all studied variables. SAS statistical software version 9.0 was used.

Results

Weather. Significant weather differences were found between the study regions, both in distribution and intensity of precipitation and temperature. In “El Parejo” orchard (Nayarit), a mean annual temperature of 21.1 °C and annual rainfall of 1287 mm were recorded. In the “Paso de Carretas” orchard (Jalisco), the mean annual temperature was 19.8 °C and annual rainfall was 717.8 mm. In “El Parejo” orchard (Michoacán), a mean annual temperature and rainfall of 19.3 °C and 1427.1 mm, respectively, were recorded (Fig. 2). The relationship between maximum solar radiation and mean environmental temperature showed Nayarit as the region with the warmest climate and greatest solar radiation. Jalisco showed a semiarid climate and the coldest region was Michoacán, albeit with solar radiation similar to that of Jalisco (Fig. 3).

Factorial analysis. The producing region (PR) and fruit development stage (DS) effects were significant \( P \leq 0.05 \) for all compounds analyzed. The PR × DS interaction was only significant \( P < 0.001 \) for TPCs and lignin (Table 1).

Influence of fruit developmental stage on the concentration of SMs and lignin in the skin. In general, the TPC concentration in the three producing regions decreased during fruit development, whereas the pigments (chlorophylls and carotenoids) and lignin increased.

TPCs decreased from initial sampling to harvest, although in the intermediate stages, differences between producing regions were observed. In Jalisco fruit, the decrease in TPC concentration in the skin was significantly smaller at harvest, whereas in Nayarit fruit, TPC concentration was smaller at S-I and Olive. In Michoacán fruit, the highest TPC concentration was found at Olive stage, whereas significantly smaller concentrations were observed at the S-II and harvest stages (Table 2).

In fruit from the three producing regions, the concentration of chlorophylls increased during fruit development, reaching its maximum value at harvest. The C<sub>N</sub> concentration in the skin of the Michoacán fruit showed no differences between developmental stages. The TCARs, like the chlorophylls, increased from Olive stage to harvest (Table 3).

In the three producing regions, lignin in the skin increased as the fruit developed. In Nayarit, the increase occurred between Olive and S-I; in Jalisco, there was no clear trend from Olive stage to S-II, but from then on, there was an upward trend in lignin concentration. In the Michoacán fruit, the...
lignin increased from Olive stage to S-III (Table 4).

Influence of producing region on the concentration of SMs and lignin in the skin. TPCs decreased during fruit development, reaching their minimum value at harvest, at which stage there was no difference between the producing regions (Fig. 4). In the Olive stage, the skin of the Michoacán fruit had a higher TPC concentration than those from Nayarit and Jalisco; in S-II and S-III, Nayarit fruit had a lower TPC concentration.

The concentration of pigments increased during fruit development, reaching its maximum value at harvest. At this stage of development, the concentration of C₄ and C₅ was higher in Nayarit fruit and lower in fruit from Jalisco (Fig. 5).

The concentration of TCARs in Olive-stage fruit was higher in fruit from Michoacán and lower in those from Nayarit; however, in S-I, the behavior was reversed. From S-II until harvest, the TPC concentration showed no differences between the producing regions (Fig. 6).

Lignin concentration increased with fruit development. In the first sampling (Olive), the skin of the Nayarit and Jalisco fruit had a higher lignin concentration than those grown in Michoacán. At harvest, the lignin concentration was higher in the Michoacán and Jalisco fruit than in those from Nayarit (Fig. 7).

Correlation analysis. Correlations between the compounds analyzed in the 'Hass' skin showed significant relationships ($P < 0.001$). TPCs in the skin correlated positively with chlorophylls ($r > 0.40$) and negatively with lignin concentration ($r = -0.66$). The concentration of carotenoids correlated negatively with chlorophylls A ($r = -0.34$), B ($r = -0.29$), and total ($r = -0.33$) (Table 5).

Discussion

The decrease observed in the TPC concentration in the skin of 'Hass' fruit during their development from the three producing regions agreed with that reported for the flesh of persimmon, where the concentration of PCs (soluble tannins) decreased during fruit development (Candir et al., 2009). A similar response occurred with the concentration of total soluble phenols in Mexican serviceberry fruit (Herrera-Hernández et al., 2013). However, it differed from what was reported by Tesfay et al. (2010) who found a slight but significant increase in TPCs in the skin of 'Hass' fruit during their development, and with findings in noni (Morinda citrifolia) where there was no change in the TPC concentration until harvest (Lin et al., 2014).

SM production varies with the different stages of plant development (Verma and Shukla, 2015). The amount and type of SMs increases or decreases as a result of the genetic expression in a species and is not the reflection of each particular plant (De-la-Cruz Chacón et al., 2013).

The color of fruits and vegetables is the result of the presence of chlorophylls, carotenoids, and anthocyanins (Lancaster et al., 1997). In avocado, pigments are important contributors to its appearance and beneficial health properties. The concentration of chlorophylls in the skin of 'Hass' fruit from the three producing regions increased during fruit development, which did not coincide with the findings reported by Yahia (2012), who indicated that during maturation and despite the color change from green to black, the concentration of chlorophylls does not change significantly. In cv. 'Hass', the color change results from an initial decrease in chlorophylls, followed by an increase in the concentration of the anthocyanin cyanidin-3-O-glucoside (Cox et al., 2004).

In the present study, the carotenoid concentration in the skin of 'Hass' fruit increased with the stage of development. This behavior coincided with that reported by Lu et al. (2009) for the concentration of these pigments in the flesh of fruit of this same cultivar as the harvest date progressed. The observed increase in the pigment concentration in the skin of 'Hass' fruit during their development coincided with that found in the fruit skin of the warm-climate avocados 'Booth 8' and 'Trinidad' at physiological maturity and consumption stages (Ceballos and Montoya, 2013).

Environmental factors such as humidity, temperature, and solar radiation are related to the levels of certain SMs, such as phenolic acids, flavonoids, chlorophylls, and anthocyanins (Alonso-Amelot et al., 2004; Pavarini et al., 2012; Verma and Shukla, 2015). This would explain the response of SMs in the present study because the concentration of total chlorophylls was lower in the 'Hass'-producing regions with lower temperatures, which coincided with Koc et al. (2010) who observed a decrease in the concentration of chlorophylls in Capsicum annuum L. exposed to low temperature.

A protection mechanism of plants against solar radiation is to decrease their chlorophyll

| Stage of development | Producing region | Nayarit | Jalisco | Michoacán |
|----------------------|-----------------|---------|---------|-----------|
| Olive                | Chlorophyll A (mg·g⁻¹ FW) | 0.084 c | 0.092 bc | 0.090 c |
|                     | Chlorophyll B (mg·g⁻¹ FW) | 0.034 c | 0.040 bc | 0.044 |
| S-I                 | Chlorophyll total (mg·g⁻¹ FW) | 0.122 c | 0.136 ab | 0.136 c |
| S-II                | Total carotenoids (mg·g⁻¹ FW) | 0.050 c | 0.056 b  | 0.060 b |
| S-III               | Olive           | 0.140 bc| 0.122 b  | 0.134 c |
| Harvest             | S-I             | 0.064 b | 0.056 b  | 0.060 b |
| Harvest             | S-II            | 0.064 b | 0.058 b  | 0.070 ab|
| Harvest             | S-III           | 0.070 a | 0.066 ab | 0.078 a |
| Harvest             | Olive           | 0.194 a | 0.158 a  | 0.182 a |
| Harvest             | S-I             | 0.156 b | 0.148 a  | 0.164 ab|
| Harvest             | S-II            | 0.140 bc| 0.116 b  | 0.146 bc|
| Harvest             | S-III           | 0.110 bc| 0.100 ab | 0.112 ab|
| Harvest             | Harvest         | 0.140 a | 0.112 a  | 0.128 a |
| Harvest             | Pr > F          | 0.056 a | 0.050 a  | 0.122 a |
| Harvest             | Pr > F          | 0.002  | 0.006    | 0.004   |
| Harvest             | Pr > F          | 0.001  | 0.004    | 0.009   |

*Means with the same letter in each column for each pigment are not significantly different (Waller-Duncan, $P \leq 0.05$).

| Stage of development | Producing region | Nayarit | Jalisco | Michoacán |
|----------------------|-----------------|---------|---------|-----------|
| Olive                | 23.28 b | 24.61 c | 15.18 c |
| S-I                 | 30.94 a | 31.44 a | 30.69 b |
| S-II                | 30.33 a | 28.60 b | 31.87 ab|
| S-III               | 30.19 a | 31.90 ab| 31.83 a |
| Harvest             | 29.88 a | 32.93 a | 32.35 a |
| Pr > F              | $<0.0001$    | $<0.0001$| $<0.0001$|

*Means with the same letter in each column are not significantly different (Waller-Duncan, $P \leq 0.05$).
concentration to prevent an energy overload that may irreversibly damage the photosynthetic system of the plant (Manrique, 2003). In the present study, the concentration of total chlorophylls in ‘Hass’ skin decreased with elevation, a response similar to that found in ‘Frailejon ochobre’ (Espeletia schultzii) leaves where populations grown at 4200 m had a lower concentration of chlorophylls than those cultivated at 3100 m (Castrillo, 2006). Even though total solar radiation was higher in Nayarit (region with the lowest elevation, 1151 m), incident ultraviolet-B radiation (315–280 nm) increases as elevation increases (Berli, 2011; Blumthaler et al., 1992). The increase in ultraviolet-B radiation favors a decrease in the concentration of chlorophylls (Swabha Takshak, 2015), which would explain the behavior of this SM in the skin of ‘Hass’ fruit produced in higher-elevation regions, such as Michoacán (1579 m) and Jalisco (2180 m).

The fruit epidermis of a plant is a very specialized tissue, which plays a critical role in the development and survival of the whole organism (Reina-Pinto and Yephremov, 2009). The skin of the avocado fruit is composed of different tissues. The sclerenchyma, comprised of lignin-accumulating cells (Schroeder, 1950), provides stiffness to the cell wall during fruit development, as well as protection because the accumulation of this compound can be induced by diseases or wounding (Whetten and Sederoff, 1995). The alteration in the production of several SMs, such as lignin, is regulated by ultraviolet-B radiation (Chen et al., 2009). As lignification is a defense mechanism, it increases as ultraviolet-B radiation stress increases, which would explain the higher lignin concentration found in the skin of ‘Hass’ fruit from Michoacán and Jalisco.

In this research, a positive correlation ($P \leq 0.001$) was found between the concentration of TPCs and chlorophylls in ‘Hass’ skin. This was the opposite of what was found by Wang et al. (2010), for warm-climate avocado cvs. Slimcado, Booth 7, Booth 8, Choquette, Loretta, Simmonds, and Tonnage, as there was no correlation between phenolics and pigments (chlorophylls and carotenoids) in mesocarp, skin and seed; however, it was agreed that there is a correlation between chlorophylls and carotenoids.

Our research showed that both the stage of development of the ‘Hass’ avocado fruit and the producing region had a significant influence on the concentrations of SMs and lignin in the skin. During fruit development, the skin showed a decrease in the concentration of PCs and an increase in the presence of chlorophylls, carotenoids and lignin. Fruit produced in the regions with a semiswarm and temperate climate were characterized by higher production of lignin and PCs, as well as a lower concentration of chlorophylls in the skin. However, the greater thickness and roughness reported for the ‘Hass’ avocado grown in Nayarit (warmest climate) could not be associated with changes on SMs. These findings demonstrate the plasticity of ‘Hass’ avocado to different climates, where the elevation and thermal gradients were the most contrasting among the three producing regions of ‘Hass’ avocado in Mexico. With abiotic stresses becoming increasingly important factors as a result of global climate change, this study provides useful information on how ‘Hass’ avocado may adapt to these changes, particularly temperature.

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Fig. 4. Influence of producing region on the concentration of total phenolic compounds (TPCs) in ‘Hass’ avocado skin at each stage of fruit development. Means with the same letter at each developmental stage are not significantly different (Waller–Duncan, $P \leq 0.05$). GAE = gallic acid equivalent.

Fig. 5. Influence of producing region on the concentration of chlorophylls in ‘Hass’ avocado skin at each stage of fruit development. Means with the same letter at each developmental stage are not significantly different (Waller–Duncan, $P \leq 0.05$).
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Table 5. Correlation coefficients (r) for the compounds analyzed in the skin of ‘Hass’ avocado fruit.

| Compounds       | CA   | CB   | CT   | TCARs | Lignin |
|-----------------|------|------|------|-------|--------|
| **TPCs**        | 0.42**| 0.59***| 0.48**| -0.66**|        |
| **Cₐ**         | 0.93***|        | 0.99**| -0.34**|        |
| **Cₐ**         | 0.96**| 0.33**|        |        |        |
| **Cₐ**         |      | 0.29**| -0.32**|        |        |

CA = chlorophyll A; CB = chlorophyll B; CT = chlorophyll total; TCARs = total carotenoids.

**P ≤ 0.001.
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