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Salting-out separation of bioactive compounds from the epicarp of 'Hass' avocado (*Persea americana* Mill.) fruit during ripening

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Abstract

The industrialization of 'Hass' avocado fruit generates waste, such as the peel or epicarp, which contains compounds that can be used. This work aimed to separate bioactive compounds from the epicarp of 'Hass' avocado fruit during ripening through salting-out or aqueous two-phase extraction (ATPE) procedures. An ATPE system was used with a mixture of trisodium citrate ($Na_3C_3H_5O(COO)_3$) and polyethylene glycol 4000 (Peg4000) at concentrations of 24.94 and 14.53 %, respectively, which allowed the recovery of 85.3 % of the soluble phenols present in the fruit epicarp. The extraction of bioactive compounds increased as ripening progressed and the highest amounts of total soluble phenols, flavonoids, anthocyanins, and condensable tannins coincided with ripeness, with values of 1 866.5, 717.6, 64.0, and 1 635.1 $\mu g \cdot mL^{-1}$, respectively, in the extraction phase. The ATPE method is a strategy that allows taking advantage of the 'Hass' avocado fruit epicarp.

Keywords: *Persea americana* Mill., aqueous two-phase extraction, phenolic compounds, postharvest.

Introduction

Avocado (*Persea americana* Mill.) fruit is known for its high nutritional content and health benefits, which are essentially due to the presence of different compounds (Forero-Doria et al., 2017) and high antioxidant activity (Villa-Rodríguez et al., 2011). 'Hass' avocado fruit is mainly consumed fresh; however, part of the production is processed to obtain traditional cuisine products such as guacamole (Woolf et al., 2013). During processing, waste is generated, such as the peel or epicarp, which has been commonly used to make compost (González-Fernández et al., 2015), although it can be put to other uses, such as the manufacture of adsorbents from the peel for dye removal (Palma et al., 2016). The use of residues resulting from industrial activity is a practice that has gained interest, because in many cases they are underutilized materials with potential for use in agricultural activity (Vázquez-Cruz et al., 2018) or as a source of high-value compounds (Arias et al., 2023). In the case of the avocado epicarp, it is a waste that contains bioactive compounds (Bowen et al., 2018; Terasawa et al., 2006), which justify the development of processes for other uses (Saavedra et al., 2017), such as the production of animal feed (Hernández-López et al., 2016), or for procedures to extract the substances of interest, due to their high antioxidant (Rodríguez-Carpena et al., 2011; Rosero et al., 2019; Wang et al., 2010) and antimicrobial (Vargas-Torrico et al., 2022) activity.

'Hass' avocado fruit is climacteric (Awad & Young, 1979) and, in addition to physical changes, undergoes compositional changes during ripening (Obenland et al., 2012; Pedreschi et al., 2016; Vekiari et al., 2004; Villa-Rodríguez et

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al., 2011). Like the pulp, the epicarp of the 'Hass' avocado fruit undergoes compositional changes as it ripens (Bowen et al., 2018; Cox et al., 2004), so the potential for utilization based on extraction of bioactive compounds from this material may vary with the physiological stage of the fruit to be assessed.

The extraction of bioactive compounds from avocado peel, for quantification purposes, has been tested using an acetone:water mixture (Rodríguez-Carpena et al., 2011; Rosero et al., 2019) or acetic acid (Wang et al., 2010). However, scaling up for industrial implementation may be limited by the requirement for large solvent volumes and long contact times (Easmin et al., 2015). Aqueous two-phase extraction (ATPE) is a procedure based on salting-out that can be used for the separation of bioactive substances such as phenolic compounds (Rodríguez-Salazar and Valle-Guadarrama, 2020), without the use of organic solvents, without heat treatment and with high scale-up potential (de Araújo et al., 2018). The ATPE method is developed through mixtures of components that can include two polymers or a polymer and a salt, which at certain concentrations generate a true solution in a homogeneous phase, but, in others, the combination causes the formation of two immiscible phases, between which the compounds of interest are separated, according to their chemical structure and affinity for interaction with the components of the system (Quintão et al., 2017). Among the most commonly used polymers is high molecular weight polyethylene glycol, due to its low-cost availability, hydrophilicity and low toxicity (Zhang et al., 2013). Regarding salts, Rodríguez-Salazar and Valle-Guadarrama (2020) indicated that the use of extracted compounds should be considered and showed that sodium citrate could be a suitable alternative. On the other hand, aqueous two-phase extraction has been shown to have the potential to efficiently extract bioactive compounds from the 'Hass' avocado fruit epicarp (Jiménez-Velázquez et al., 2020). In this context, the aim of this work was to evaluate the separation of bioactive compounds from 'the Hass' avocado fruit epicarp throughout ripening using aqueous two-phase extraction procedures.

Materials and methods

Plant material

'Hass' avocado fruit weighing 187.76 (± 30.89) g, at physiological maturity, were harvested from the Tetela del Volcán region of Mexico (18° 53´ 35" N, 98° 43´ 47" W), located at 2 231 m above sea level and characterized by a temperature variation between 9.8 and 22.9 °C.

Experimental set-up

The fruit were placed in an isolated environment at 25 (±1) °C with relative humidity of 40 (±1) %. Ten fruits were selected daily for 10 d of storage for firmness and color assessment. Firmness was measured on two sides of the equatorial zone with a texture analyzer (SM-100N-168, Ametek and Chatillon, Florida, USA) fitted with a conical attachment with a diameter of 3.4 mm at the base and a length of 5 mm. A compression force measurement routine was applied with a test speed of 5 $mm \cdot s^{-1}$ and a deformation distance of 5 mm. The mean of the measurements was obtained for each fruit and the results were expressed in Newton (N). Color was measured in the epicarp with a colorimeter (X-rite mod. 3690®, USA) and expressed as lightness (L*), hue angle (H*) and chroma (C*) (McGuire, 1992). Subsequently, the epicarp was removed from the fruit, lyophilized, ground and stored in resealable plastic bags at -20 °C, to be used as source material for the separation of phenolic compounds, flavonoids, anthocyanins and condensable tannins, with an aqueous two-phase extraction (ATPE) procedure. Likewise, the material corresponding to physiological maturity and ripeness was subjected to proximate analysis using AOAC (1990) procedures.

Separation potential of bioactive compounds

Based on the recommendation of Jiménez-Velázquez et al. (2020), aqueous solutions containing 20 g of trisodium citrate and polyethylene glycol 4000 (Peg4000) were prepared at concentrations of 24.94 and 14.53 %, respectively. Then, 0.5 g of lyophilized epicarp from physiologically mature fruit was incorporated into the solutions. The mixture was homogenized in an Ultra Turrax T25 instrument for 5 min at 10 000 rpm and mixed in a Vortemp 56 shaking incubator (ThermoFisher Scientific, USA) for 10 min at 10 000 rpm. The solutions were left standing for 12 h to allow the formation of two-phase systems. The phases were separated and volume (V), concentration of total soluble phenols (c_{tsp}), volume ratio (V_r), partition coefficient (K) and separation yield (Y_{tsp}) were measured in each one, using the calculations described in Equation (1) (Jiménez-Velázquez et al., 2020), where the subscripts t and b refer to the upper and lower phases of the systems, respectively.

$$V_r = \frac{V_t}{V_b}; \qquad K = \frac{(c_{tsp})_t}{(c_{tsp})_b}; \qquad Y = \frac{(c_{tsp})_t V_t}{(m_{tsp})_0}$$

Separation of bioactive compounds during storage

Mixtures of Peg4000 and trisodium citrate were prepared at concentrations of 24.94 and 14.53 %, respectively. The systems were incorporated with lyophilized epicarp from each of the days evaluated during postharvest storage in a 2.5 % proportion. All mixtures were subjected to homogenization, shaking and resting under the same conditions as described above. In each case, the upper phase of the systems was recovered and the content of total soluble phenols, flavonoids, anthocyanins and condensable tannins was evaluated.

Concentration of bioactive compounds

The content of total soluble phenols (*tsp*) was determined using the Folin-Ciocalteu (FC) reagent method (Singleton and Rossi, 1965), where 250 µL of FC was reacted with 100 µL of sample in test tubes for six min. Subsequently, the mixture was neutralized with 1.25 mL of a Na_2CO_3 solution (19 % w/v) and the volume was adjusted to 3.0 mL with distilled water. The mixtures were vortexed and placed in the dark for 90 min to achieve stabilization. Centrifugation (Hermle Z200 equipment, Labortechnik, Germany) was applied at 13 000×g for 10 min to remove turbidity and absorbance was determined in a UV-Vis spectrophotometer (DR 500 UV-vis HACH, USA) at 760 nm. To determine the concentration, a gallic acid standard curve was performed and the *tsp* content was expressed as µg gallic acid equivalents per milliliter (µg GAE·mL^{1}).

A calibration curve with 3 000 ppm of (+)-catechin (Kubola & Siriamornpun, 2011) was used to quantify flavonoids (*fla*). The necessary dilutions for each extract treatment were made with distilled water. Subsequently, a 0.5 mL aliquot was taken and dissolved in 2 mL of distilled water. Then, 0.15 mL of 5 % $NaNO_2$ was added and the sample was allowed to stand for 6 min in the dark. Next, 0.15 mL of 10 % $AlCl_3$ was added and it was allowed to stand for 6 min in the dark. After that, 2 mL of 4 % NaOH and 0.2 mL of distilled water were added to bring the volume to 5 mL. Finally, the samples were read at 510 nm in a spectrophotometer (DR 500 UV-vis HACH, USA). The results were expressed in µg catechin·mL^{-1} (µg EPC·mL^{-1}).

To measure the concentration of anthocyanins (*ant*), a cyanidin chloride standard was used, with which a calibration curve was prepared for a concentration varying between 5 and 30 µg·mL^{-1}. The absorbance of the samples was then measured in a spectrophotometer (DR 500 UV-vis HACH, USA), at 535 nm. The results were reported in µg cyanidin chloride equivalents per milliliter (μg_{cc} ·mL^{-1}).

To determine the condensable tannin (*tan*) content, the method described by Price et al. (1978) was used with some modifications. Twenty µL of the upper phase obtained from the ATPE systems was taken, added to 180 µL of methanol and shaken. Then, 1.2 mL of 4% w/v vanillin (analytical grade, Merck) in methanol (analytical grade, Merck) was added. Finally, 600 µL of concentrated HCl was added and protected from light for 30 min. Absorbance was measured at a wavelength of 500 nm. A calibration curve was prepared with (+)-catechin (EPC) at a concentration ranging from 2 to 35 µg·mL^{-1}. The content of condensable tannins was expressed in µg catechin equivalents per milliliter (µg EPC·mL^{-1}).

Data analysis

The work had time as the only factor of variation. The data on pulp firmness, epicarp color and separation yields of soluble phenols, flavonoids, anthocyanins and condensable tannins from the epicarp were subjected to analysis of variance and comparison of means routines with Tukey's test, using a significance level of 0.05. Likewise, the results of the proximate analysis of the epicarp of 'Hass' avocado fruit were compared between the physiological maturity and ripeness conditions (Table 1). All routines were performed in triplicate.

Results and discussion

Firmness

The fruit presented initial firmness of 39.27 (± 2.08) N and during storage this variable decreased with a sigmoidal behavior, where the most visible changes occurred between 4 and 7 d, to reach final values of 1.10 (± 0.04) N (Figure 1A). 'Hass' avocado fruit is climacteric and firmness reduction is a characteristic of ripening, which has been reported by Sierra et al. (2019) and Valle-Guadarrama et al. (2013) at temperatures of 20-21 ºC. The decreased firmness in fruit and vegetable products is related to the degradation of pectin and hemicellulose, present in the cell wall, by enzymatic action (Wang et al., 2018).

Color

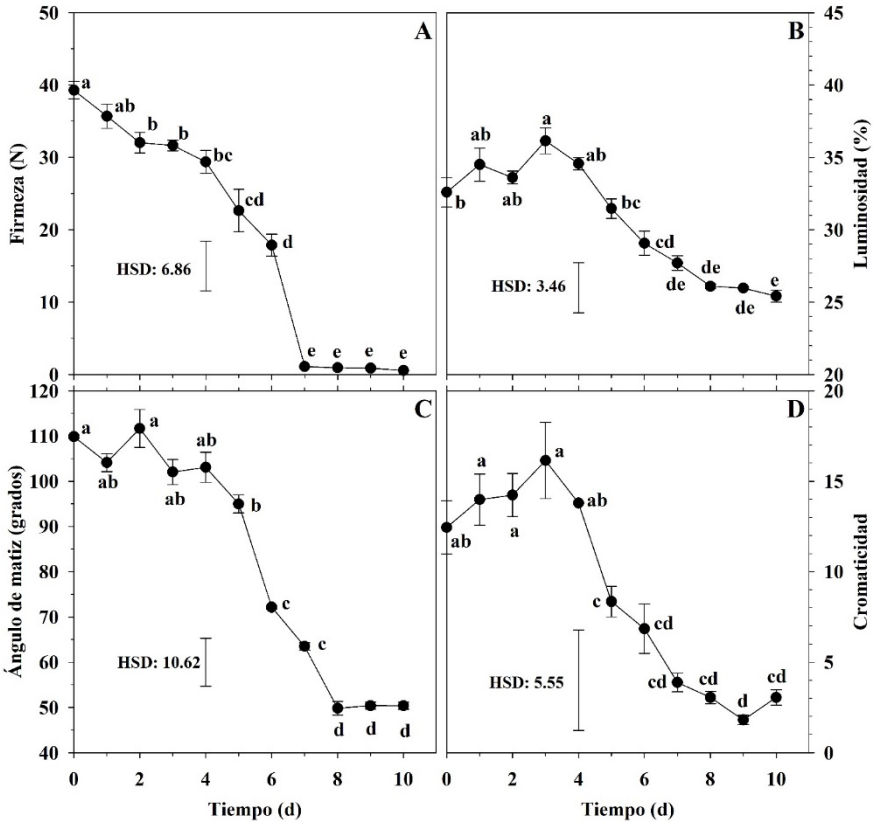
The lightness (L*), hue angle (H*) and chroma (C*) of the epicarp remained in ranges with non-significant differences ($P > 0.05$), between 32.6-36.2 %, 102.1-111.7° and 12.4-13.8, respectively, during the first four days of storage. However, between four and eight days, the three attributes experienced a significant decrease ($P \leq 0.05$),

Table 1. Physicochemical parameters of 'Hass' avocado fruit epicarp at ripeness and physiological maturity (dry basis).

Determinations (%)	Physiological maturity PM	Ripeness R
Moisture	75.70 b ± 0.05	76.31 a ± 0.37
Ash	0.59 b ± 0.07	0.88 a ± 0.05
Crude protein	7.57 b ± 0.006	8.36 a ± 0.32
Ether extract	9.57 a ± 0.51	4.83 b ± 0.90
Crude fiber	1.70 b ± 0.11	3.31 a ± 0.68

Means followed by the same letter within rows are not statistically different based on Tukey's test ($P \leq 0.05$) ± standard deviation.

Figure 1. Changes in firmness and epicarp color of 'Hass' avocado fruit throughout postharvest ripening. Different letters indicate significant difference (Tukey, 0.05). HSD: Honest Significant Difference. Error bars correspond to standard errors.



with a practically linear behavior from 34.6 to 26.1 % in L*, from 103.1 to 49.8° in H* and from 13.8 to 3.05 in C*. Finally, in the eight-to-ten-day period these attributes remained in ranges around average values of 25.8 %, 50.2 ° and 2.6, respectively, with no significant differences (P > 0.05) (Figures 1B, 1C and 1D). The ripening of 'Hass' avocado fruit is accompanied by a change in epicarp color and is typically characterized as a darkening. This behavior has been explained in terms of a reduction in chlorophyll content and synthesis of pigments, such as anthocyanins, as the postharvest period progresses (Cox et al., 2004). The values were similar to those reported by Salcedo et al. (2018) at a storage temperature of 10 °C for 20 d and Sierra et al. (2019) during 20 d of storage at a temperature of 21 °C. On the other hand, Arpaia et al. (2018) showed final storage values for 11 d of 28.29, 5.45 and 67.20 of L*, C* and H*, respectively, at a temperature of 25 °C.

Proximate analysis

The bromatological composition of the fruit epicarp was different ($P \leq 0.05$) between physiological maturity (PM) and ripeness (R). After moisture, the major components were ether extract at PM and crude protein at R. The nitrogen-free extract was obtained by percent difference with the ash, crude fiber, ether extract, crude protein and moisture percentages, and had an average value of 4.87 % at physiological maturity and 6.31 % at ripeness, which indicated that it was the fourth most important fraction in the fruit epicarp. Daiuto et al. (2014) evaluated the proximate composition of the 'Hass' avocado fruit epicarp, but reported lower values of ether extract (2.18 %) and protein (0.17 %), although the crude fiber level was similar (1.29 %). The composition of avocado residues varies between cultivars and sometimes within the same cultivar, which may be due to factors that influence the composition of the fruit during its development, including the region of avocado production, climate, elevation, precipitation, and genetics, among others (Araújo et al., 2018).

Separation potential of bioactive compounds with ATPE

The mixture of polyethylene glycol 4000 (Peg4000) and trisodium citrate at concentrations of 12.23 and 15.51 %, respectively, formed two immiscible phases. This occurred due to the salting-out phenomenon (Sadeghi & Jahani, 2012). At the concentration of sodium citrate used, the interaction of ions in solution with water was high enough to cause a reduction in the interaction of the polymer chains with the solvent, which translated into a decrease in their solubility, resulting in the exclusion of

the polymer from the solution and the formation of two immiscible phases. Thus, after a rest time, an upper phase with a volume of 3.6 (±0.3) mL and a lower phase with a volume of 9.6 (±0.1) mL were obtained, which generated a volume ratio (V_r) of 0.38 (±0.03), i.e. the upper phase was significantly smaller than the lower phase. The incorporation of lyophilized epicarp from physiologically mature fruit caused a concentration of total soluble phenols (c_{tsp}) of 865.12 (±18.34) $\mu g \cdot mL^{1}$ in the upper phase and 7.70 (±0.8) $\mu g \cdot mL^{1}$ in the lower phase, which generated a partition coefficient (K) of 112.4 (±6.3), i.e. 97.67 % (±0.25 %) of such compounds migrated to the upper phase. On the other hand, the concentration found in the plant material was 7 300 (±1,890) $\mu g \cdot g^{1}$. In this sense, since 0.5 g of lyophilized epicarp was added, the systems were incorporated, on average, with 3 650.0 µg of soluble phenols [$(m_{tsp})_0$]. Thus, considering the volume of the upper phase, the amount of phenolic compounds present in it was 3 114.4 µg, i.e. 85.3 % of the amount present in the lyophilized epicarp was recovered in this phase, which was considered as the separation potential (Y) of bioactive compounds of the aqueous two-phase extraction system used.

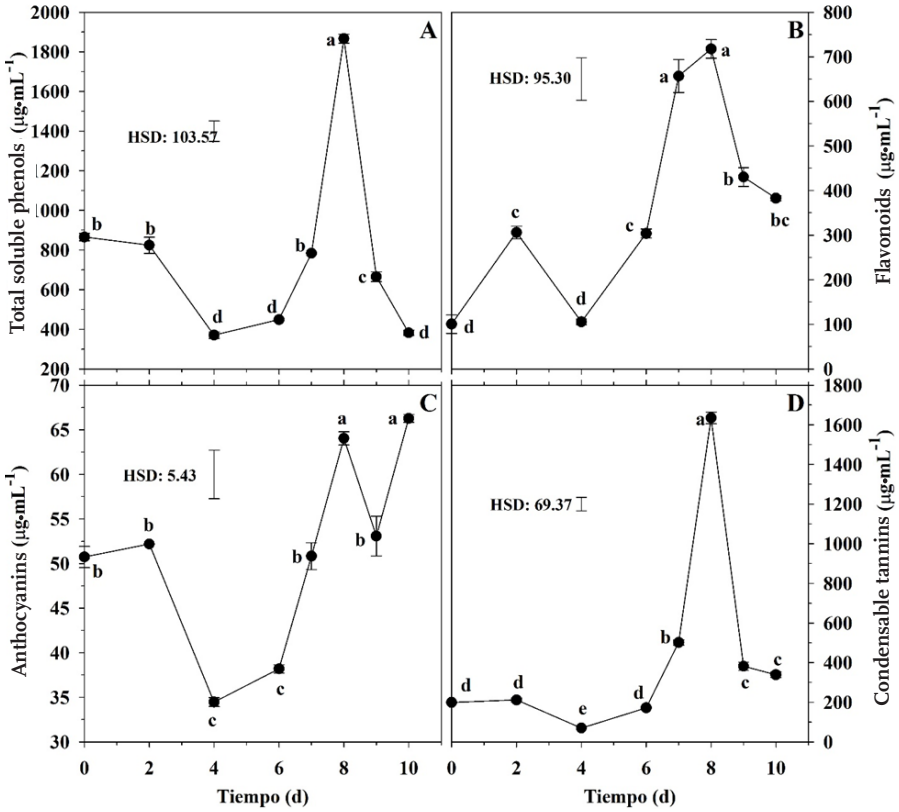
Separation of bioactive compounds during storage

ATPE systems were used to separate soluble phenols (*tsp*), flavonoids (*fla*), anthocyanins (*ant*) and condensable tannins (*tan*) from the epicarp of fruit separated on different days throughout the 10-d storage. The concentration found in the upper phase of the systems decreased ($P \leq 0.05$) in the first four days of storage, from 865.1 to 370.3 $\mu g \cdot mL^{1}$ in *tsp*, from 50.7 to 34.5 $\mu g \cdot mL^{1}$ in *ant* and from 198.7 to 70.0 in *tan* (Figure 2). The *fla* concentration showed variability between 100.3 and 306.1 $\mu g \cdot mL^{1}$ in that period, but without a clear trend. However, in the period from four to eight days of storage, the concentration of compounds in the upper phase of the systems increased significantly ($P \leq 0.05$) until reaching maximum values of 1 866.5 (±22.6), 717.6 (±20.8), 64.0 (±0.74) and 1 635.1 (±29.4) $\mu g \cdot mL^{1}$ in *tsp*, *fla*, *ant* and *tan*, respectively. Subsequently, the cases of *tsp*, *fla* and *tan* experienced a decrease in the concentration found in the period from eight to ten days of storage, while the anthocyanin content remained with values close to the maximum reached.

It is well established that, in addition to physical changes such as firmness and color, avocado fruit pulp undergoes increases in dry matter, oil and protein, as well as decreases in total sugars (Pedreschi et al., 2016; Vekiari et al., 2004). Likewise, changes have been characterized in the profiles of fatty acids (Pedreschi et al., 2016; Vekiari et al., 2004) and volatile compounds (Obenland et al., 2012) and an increase in phenolic compounds in general has even been determined, but a decrease in flavonoids (Villa-Rodríguez et al., 2011). Likewise, the fruit epicarp undergoes changes in its chemical composition, and data from the present work show that the contents of total soluble phenols in general, and of flavonoids, anthocyanins and condensable tannins in particular, changed with ripening. The presence of phenolic compounds in the avocado fruit epicarp has been reported in other studies. In this regard, the presence of catechin, epicatechin, six quercetin derivatives, procyanidin dimers, as well as trimers, tetramers, pentamers and hexamers has been determined (Rosero et al., 2019; Wang et al., 2010). It has also been reported that the fraction of bioactive compounds with the highest antioxidant activity has been condensable tannins (Rosero et al., 2019). Likewise, Bowen et al. (2018) investigated changes in compounds in the epicarp of avocado fruit associated with an antifungal effect, where they determined that the concentration of persin, a water-insoluble compound with a fatty acid-like structure, decreases with ripening in parallel to the reduction of epicatechins.

The dark color of the 'Hass' avocado fruit epicarp at ripeness is caused by the presence of anthocyanins (Cox et al., 2004). Although these compounds can be used as natural colorants (Jamei & Babaloo, 2017), the concentration found of them was actually low compared to other materials such as some strawberries (Oladzadabbasabadi et al., 2022). To verify this situation, samples identified with high and low anthocyanin concentrations corresponding to physiological maturity and ripeness stages, respectively, were selected. In both cases, a 1:15 dilution with methanol was performed and a scan from 700 to 200 nm was obtained in a spectrophotometer (DR 500 UV-vis HACH, USA). Both types of samples showed similar spectra. The structure of anthocyanins is pH sensitive (Lee et al., 2005). On this basis, the pH of the samples was reduced with HCl to values between 0 – 1 and the color of the samples changed only to a slightly pinkish tone, with absorbance values between 0.06 and 0.08 at a 530 nm wavelength, indicating a very low concentration of anthocyanins, but a very high concentration of other types of compounds. In this regard, the results showed that, although the epicarp of 'Hass' avocado fruit cannot be considered an important source of anthocyanins, it is an important source of phenolic compounds in general and of condensable tannins in particular, in addition to being a moderate source of flavonoids. Given the high antioxidant activity of this type of compound (Martins et al., 2016), the epicarp of fruit can have a high commercial value and, since it is an underutilized product of fruit industrialization, its use can contribute to increasing the added value of this material. The content of phenolic compounds changes with the maturity stage, which is why Dong et al. (2019) pointed out that it is necessary to identify the physiological condition that optimizes the use of this type of compound. In this regard, the results of the present work indicated that the highest concentrations of the bioactive compounds evaluated coincide with the moment when the fruit reaches ripeness, which constitutes an advantage for companies that industrialize 'Hass' avocado fruit, where the required physiological condition coincides with this stage. However, since the concentration of bioactive compounds decreases in the epicarp during the last days of storage, it is necessary to avoid over-ripening of the fruit in order to get the maximum benefit from them.

Figure 2. Changes in the phytochemical composition of the 'Hass' avocado fruit epicarp during postharvest ripening. Different letters indicate significant difference (Tukey, 0.05). HSD: Honestly significant difference. Error bars correspond to standard errors.



Conclusions

The 'Hass' avocado fruit epicarp is rich in soluble phenols, flavonoids and condensable tannins, although its anthocyanin content is low. The content of soluble phenols, flavonoids, anthocyanins and condensable tannins is modified in the 'Hass' avocado fruit epicarp during ripening in the postharvest period, with maximum values reached coincident with ripeness in an ambient thermal condition. Aqueous two-phase extraction is a useful procedure to separate bioactive compounds from the 'Hass' avocado epicarp obtained from different stages of fruit maturity.

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