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Content of bioactive compounds and their contribution to antioxidant capacity during ripening of pineapple (*Ananas comosus* L.) cv. Esmeralda

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Summary

Pineapple (*Ananas comosus* L.) cv. Esmeralda is a commercially important fruit with many bioactive compounds like vitamin C, β-carotene, phenolic compounds and flavonoids, which have been reported only for fruits of commercial maturity. Our objective was to evaluate changes in concentration of main pineapple bioactives, their contribution to total antioxidant capacity and enzyme activities of phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POD) during pineapple ripening. Fruits were grouped into four ripening stages (RS) according to their weight, size and percentage of yellow skin color (RS1: 100% green, RS2: up to 30% yellow, RS3: 30% - 75% yellow, RS4: 75% - 100% yellow). Vitamin C content initially increased, and decreased at RS4; β-carotene, phenolics and antioxidant capacity increased gradually. Phenolics contributed over 40% of antioxidant capacity, followed by vitamin C and β-carotene. Major phenolic compounds identified were gallic acid, catechin and epicatechin. PAL and POD activity increased with ripening and correlated with concentration of phenolics. No PPO activity was quantified. We concluded that ripening of pineapple cv. Esmeralda alters the concentration of bioactive compounds. Phenolic compounds, particularly gallic acid, exert the most antioxidant capacity during all RS, even if other compounds have higher concentrations.

Introduction

Pineapples (*Ananas comosus* L.) are tropical fruits that are highly regarded in various cultures because of their desirable organoleptic qualities. Their consumption is recommended, along with fruits and vegetables in general, because epidemiological evidence shows that they have a positive effect on human health. These effects are attributed to phenolics, vitamin C and β-carotene, among others, which are highly bioactive, partly because of their high antioxidant capacity. Antioxidant capacity is not an absolute value, it depends on the identity and quantity of the molecules present, among other factors. Furthermore, the individual concentration of each bioactive is also not absolute, it depends on fruit cultivar and on ripening stage (RS) of the fruit (Wall-Medrano et al., 2016).

In addition to the concentration of bioactive compounds, activity of various enzymes also changes during the ripening process. Phenylalanine ammonia lyase (PAL, E.C. 4.3.1.24) catalyzes phenylalanine into ammonia and *trans*-cinnamic acid, which initiates the synthesis of polyphenols in general and flavonoids in particular through the phenylpropanoid pathway (Ganapathy et al., 2016). Polyphenol oxidase (PPO, E.C. 1.14.18.1) oxidizes the hydroxyl groups of polyphenols into quinone moieties, a process responsible for enzymatic browning. Peroxidase (POD, E.C. 1.11.1.7) is involved in the synthesis of lignin and in the stress response, it also regulates the concentration of hydrogen peroxide (Raimbault et al., 2011).

Antioxidant capacity and activities of PAL, PPO and POD have been reported mainly in pineapples of commercial maturity, while the changes that take place during the ripening process have not been thoroughly documented. Because of this, the purpose of this study was to evaluate the changes in concentration of bioactive compounds, their contribution to total antioxidant capacity and changes in enzyme activities during ripening of pineapple cv. Esmeralda.

Materials and methods

Samples

Pineapples cv. Esmeralda were harvested at different RS from commercial fields in Ruiz, in the state of Nayarit, Mexico (21.95° N, 105.14° W), and transported the same day to the Tepic Technological Institute. Upon arrival, fruits were sanitized with 200 ppm chlorinated water for 3 min and dried at room temperature. Fruits free of defects and mechanical damage were grouped into four RS according to their weight, size and percentage of yellow skin color (RS1: 100% green, RS2: up to 30% yellow, RS3: 30% to 75% yellow, RS4: 75% - 100% yellow). Five fruits from each RS were selected for physiological characterization. In addition, samples of pulp (discarding the core) from each RS were frozen at -80 °C and subsequently lyophilized and stored at -35 °C until analyzed.

Physicochemical parameters

Color (L*, a*, b*) was measured on the surface of pineapple pulp using a Minolta CR-300 colorimeter (Minolta). Titratable acidity (TA) and pH were evaluated according to the AOAC official method (Association of Official Analytical Chemists, 1990). TA was expressed as % citric acid. Total soluble solids (TSS) were measured directly in an Abbe 10450 digital refractometer (American Optical) at 25 °C, results are reported in °Brix. Juice content was determined as described by Carlin et al. (1990) and expressed as %.

Ascorbic acid (AA)

Ascorbic acid (AA) was analyzed using a Varian Solvent Delivery System, pump Model 9012 and a Rheodyne Model 7125 injector (Rheodyne Inc.) fitted with a 10 µL loop and a Varian Model 9020 UV-VIS absorbance detector. AA was determined according to Doner and Hicks (1981) with slight modifications. Lyophilized fruit tissue (0.2 g) was homogenized with an Ultra Turrax T25 basic homogenizer (IKA) for 1 min with 10 mL of an aqueous solution containing 30 g/L of metaphosphoric acid and 80 mL/L of acetic acid. The homogenate was filtered and centrifuged for 15 min at 9,400 g (Beckman Coulter). The supernatant was filtered (0.22 µm) and analyzed by HPLC with a waters-NH_2 type Bondapak (Waters) analytical column (3.9 × 300 mm, 10 µm), and a mobile phase of acetonitrile/KH_2PO_4 (75:25 v/v) with a flow rate of 1.5 mL/min. AA was detected by ultraviolet absorption at 268 nm and its concentration calculated using a standard curve. Results are expressed as mg of AA/100 g of fresh weight (FW).

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β-carotene

β-carotene was measured as described by Mejia et al. (1988). Lyophilized fruit tissue (0.2 g) was homogenized for 1 min with 10 mL of tetrahydrofuran, containing 0.4% butylated hydroxytoluene (BHT). The mixture was centrifuged for 15 min at 6,700 g, filtered (0.22 μm) and analyzed by HPLC using a Microsorb RP-C_{18} column (4.6 mm × 10 cm, 3 μm) with a 3 cm guard column (Supelco). Mobile phase was acetonitrile/methanol/tetrahydrofuran (58:35:7 v/v/v) with a flow rate of 1.0 mL/min. β-carotene was detected by its absorption at 460 nm, it was identified by comparing its retention time with a standard and its concentration calculated with a standard curve. Results are expressed as μg of β-carotene/100 g of FW.

Extraction of bioactive compounds

Hydrophilic extracts were obtained according to Shivashankara et al. (2004) with some modifications. Lyophilized fruit tissue (1 g) was homogenized with 20 mL of 80% methanol at room temperature. The homogenate was sonicated for 30 min (Branson) and centrifuged at 9,400 g for 15 min at 4 °C. The supernatant was collected and the precipitate was re-extracted as described. The two supernatants were mixed and filtered using Whatman No.1 filter paper. The extracts were stored at -30 °C for subsequent determination of total phenolics, flavonoids and antioxidant capacity by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Trolox equivalent antioxidant capacity (TEAC) assays. The extraction process was done in triplicate for each RS.

Extraction of lipophilic components was performed as described by Huang et al. (2002). Lyophilized fruit tissue (1 g) was homogenized in 10 mL of acetone at room temperature and centrifuged at 9,400 g for 15 min at 4 °C. The supernatant was collected and an aliquot was diluted with 7% methylated β-cyclodextrin solvent (w/v) made in a 50% acetone-water mixture (v/v) and shaken for 1 h at room temperature. Extracts were stored at -30 °C until used for DPPH and TEAC assays. Extraction was done in triplicate for each RS.

Total phenolics and flavonoids

Total phenolics were measured as described by Singleton and Rossi (1965) with the Folin-Ciocalteu reagent, using a standard curve of gallic acid and expressed as mg of gallic acid equivalents (GAE)/ 100 g of FW.

Total flavonoid content was determined as described by Zhishen et al. (1999) with the aluminum chloride method, using a standard curve of quercetin. Results are expressed as mg of quercetin equivalents (QE)/100 g of FW.

Antioxidant capacity

DPPH assay was determined according to Brand-Williams et al. (1995). Radical scavenging activity (RSA) is expressed as inhibition percentage.

TEAC value was determined according to Re et al. (1999). Results are expressed as μmol of Trolox equivalents (TE)/100 g of FW.

Phenolic profile

Identification of phenolic compounds was performed as described by Rivera-Pastrana et al. (2010). Lyophilized fruit tissue (0.5 g) was homogenized in 20 mL of 80% methanol, sonicated for 30 min at 30 ºC, centrifuged at 8,000 g for 15 min at 5 °C and filtered through Whatman No. 1 filter paper. For alkaline hydrolysis, 10 mL of 4 M NaOH were added to phenolic extracts and incubated for 4 h in the dark at room temperature. Extracts were then acidified to pH 2.0 with 4 M HCl. Acidified solutions were then extracted twice with 20 mL ethyl acetate. Extracts were dried by rotary evaporation (Büchi) at 35 °C. Samples were resuspended in 10 mL of 80% methanol, filtered and injected into an HP 1100 series HPLC system (Hewlett-Packard) equipped with a diode array detector (DAD). The HPLC system was equipped with an Xterra RP-C_{18} reverse phase column (4.6 mm × 250 mm, 5 μm) kept at 25 °C. The mobile phase was 1% formic acid (A) and acetonitrile (B), elution gradient was 2 to 100% (B) in 40 min at a flow rate of 0.5 mL/min. Injection volume was 20 μL. External standards were used to identify individual phenolics.

Contribution of individual bioactive compounds to total antioxidant capacity

Contribution of individual bioactive compounds to total antioxidant capacity was calculated by the DPPH assay at the quantified concentration at each RS. Percentages were calculated as follows:

$$\text{Contribution to TAC} = \left(\frac{TAC - DPPH \text{ inhibition percentage}}{TAC} \right) \times 100 \qquad (1)$$

where TAC: total antioxidant capacity.

Phenolic compounds were considered a single contributor, but to further understand the contribution of each phenolic, the antioxidant capacity of each identified compound was also assayed in increasing concentrations up to 0.5 mg/mL.

Phenylalanine ammonia lyase (PAL) activity

PAL activity was measured using the procedure of Ke and Saltveit (1986) with slight modifications. For each replicate, 1 g of lyophilized tissue was mixed with 0.2 g of polyvinylpyrrolidine (PVPP) and homogenized in 30 mL of 50 mM cold borate buffer (pH 8.5, containing 400 μL/L of β-mercaptoethanol) at low speed to a uniform consistency. The extracts were then filtered through 4 layers of cheesecloth and centrifuged at 20,000 *g* for 15 min at 4 °C. After centrifugation, a clear supernatant was collected and incubated for 5 min in a 40 °C water bath. 100 mM of L-phenylalanine were then added as substrate. Activity was measured after 0 and 1 h of incubation in a 40 °C water bath. PAL activity is expressed as μmoles of *trans*-cinnamic acid/h g of FW, calculated using a standard curve of *trans*-cinnamic acid in borate buffer.

Polyphenol oxidase (PPO) activity

Because a crude extract of PPO may contain interfering substances, the enzyme was extracted with acetone as described by Flurkey and Jen (1978) and Das et al. (1997). 2 g of acetone powder was suspended in 10 mL of 0.01 M phosphate buffer, pH 7 with 20 μL of 0.1 M phenyl methyl sulphonyl fluoride (PMSF). The suspension was stirred for 60 min at 4 °C and centrifuged at 0 °C for 30 min at 6,700 g. The supernatant was filtered and kept at 25 °C for 60 min. The reaction mixture contained 150 μL of extract and 850 μL of 0.5 M catechol in sodium citrate buffer, pH 5. Changes in absorbance (420 nm) were recorded every 10 s up to 60 s. Each sample was run in triplicate. A unit of enzyme activity changes 0.001 absorbance units/ min mg protein.

Peroxidase (POD) activity

POD activity was determined as described by Pérez-Tello et al. (2009). The enzyme was extracted from 0.2 g of acetone powder from pineapple flesh and homogenized with 5 mL of 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM β-mercaptoethanol. The mixture was stirred for 20 min at 4 °C, centrifuged for 30 min at 8,000 g and 4 °C and recovered supernatant was decanted. POD activity was measured at 470 nm for 2 min at 30 °C in 2.15 mL of reaction solution

with 10 mM sodium acetate (pH 5.3) containing 0.5% guaiacol, 0.25 mL of 0.1% hydrogen peroxide and 0.1 mL of enzyme extract. POD activity is reported as the decomposition of 1 μmol of guaiacol/min mg protein.

Statistical analysis

Analysis of variance (ANOVA) and Duncan multiple comparison tests were used to evaluate differences between RS. All analyses were performed on the NCSS software (NCSS, LLC) and considered significant when $p < 0.05$.

Results and discussion

Physicochemical characterization

Tab. 1 lists the physicochemical parameters analyzed at different RS of pineapple cv. Esmeralda. Except for pH, all variables showed significant differences throughout the ripening process. The most important changes occurred in TA and L* and b* values of flesh color. Citric acid content ranged from 0.47 to 1.02% in RS1 and RS4, respectively, while L* value decreased, resulting in a loss of brightness and an increase in yellow color (b*) characteristic of the pineapple flesh during ripening.

Juiciness increased gradually as ripening progressed. This is associated with a lower water holding capacity of the fruit as a result of chemical changes of the major cell wall components (cellulose, hemicellulose and pectin) that occur during ripening.

TSS and TA values were within the established range (TSS = 10 - 18% and acidity = 0.4 - 1.2% citric acid) for pineapples of edible quality (Kader, 2009). Similar values were reported for Cayenne, Gold and Premium Select pineapples (Marrero and Kader, 2006; Montero-Calderon et al., 2010).

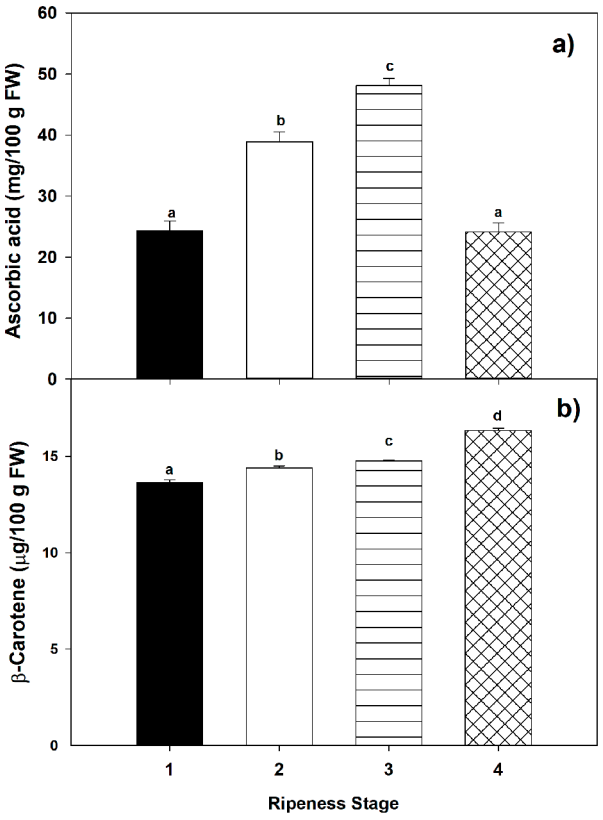

Fig. 1: Changes in ascorbic acid (a) and β-carotene (b) concentration in pineapple cv. Esmeralda at four ripening stages (RS). Different letters indicate significant differences ($p < 0.05$), n = 12.

Ascorbic acid (AA)

Fig. 1 shows the AA content (1a) and β-carotene content (1b) of pineapple cv. Esmeralda. AA gradually increased until RS3 and then decreased at RS4. The information available regarding bioactive compounds and antioxidant capacity is for commercial maturity fruits, but not the changes that occur during ripening. For comparison, in this study we took into account the values presented at RS3, which is generally the RS at which the fruit is commercialized. AA content of Esmeralda pineapple is superior to values reported for Gold, Phulae and Nanglae pineapples, with 33.3, 18.8 and 6.5 mg AA/100 g FW, respectively (Kongsuwan et al., 2009; Montero-Calderon et al., 2010), and lower than that reported by Marrero and Kader (2006) for Premium Select pineapples (78.9 mg AA/100 g FW).

The increase of AA during early RS may be due to increasing sugar concentration, predominant sugars in pineapple are sucrose, glucose and fructose, the latter two are AA precursors. However, the decrease observed in RS4 can be explained by an increased energy consumption of metabolic processes, or as an indication of increased free radical production, so that AA could act synergistically by regenerating phenolic compounds, resulting in its decreased concentration.

β-Carotene

Concentration of β-carotene increased gradually with ripening, the maximum concentration occurred at RS4. Studies by Kongsuwan et al. (2009) in Phulae and Nanglae pineapple of commercial maturity

Tab. 1: Physicochemical characterization of pineapple cv. Esmeralda at four ripening stages (RS)

Physicochemical parameter	RS1	RS2	RS3	RS4
total soluble solids (TSS, %)	14.86±0.41^{a}	16.53±0.51^{c}	16.40±0.29^{bc}	16.12±0.06^{b}
titratable acidity (TA, % citric acid)	0.47±0.03^{a}	0.63±0.01^{b}	0.88±0.06^{c}	1.02±0.01^{d}
juiciness (%)	3.01±0.01^{a}	4.03±0.02^{ab}	4.73±0.04^{ab}	5.57±0.06^{b}
pH	3.82±0.02^{a}	4.03±0.01^{a}	3.93±0.02^{a}	3.91±0.02^{a}
Color				
L*	68.52^{d}	66.60^{c}	64.66^{b}	62.59^{a}
a*	-3.66^{c}	-4.84^{a}	-4.57^{b}	-4.88^{a}
b*	17.78^{a}	24.37^{b}	26.17^{c}	28.99^{d}

RS: ripening stage. Different superscript letters in the same row indicate significant differences ($p < 0.05$), n = 12.

show that β-carotene content in those cultivars is lower than that found in Esmeralda pineapple at RS3, however this value is two times lower than what was reported by Marrero and Kader (2006) in Premium Select pineapples.

Increased β-carotene concentration may indicate that carotenoids are acting together with chlorophyll in photosynthesis. Its function is to assist in photon absorption and extend the range of light absorbed by photosynthetic pigments, especially in the blue-green range, which is transferred to the photosynthetic reaction centers. When light absorption is greater than photosynthesis capacity, excitation energy leads to the formation of chlorophyll and reactive singlet oxygen (Kopsell and Kopsell, 2010). During ripening, ethylene degrades chlorophyll, promoting mevalonic acid synthesis, a precursor of carotenoids, which dissipate excess energy to inhibit oxidative damage, stabilizing singlet oxygen and leading to the formation of yellow-orange pigments.

Phenolic compounds

Fig. 2 shows the total phenolic content (2a) and flavonoid content (2b) of pineapple cv. Esmeralda. Total phenolic content increased significantly during ripening and reached a maximum at RS4. Studies by Ayala-Zavala et al. (2010) and Montero-Calderon et al. (2010) in Cayenne and Gold pineapple showed that total phenolic content found in these two varieties is up to 40% lower than that found in Esmeralda pineapple at RS3. These changes could be related to cultivar, agronomic practices, post-harvest handling and treatments.

Flavonoid content increased gradually from RS1 to RS4. This increase may be due to variation in the content of precursors during maturation; starch, abundant in non-climacteric fruits, is hydrolyzed into sugars very quickly, and favors the synthesis of shikimic and mevalonic acids, precursor of phenylpropanoids.

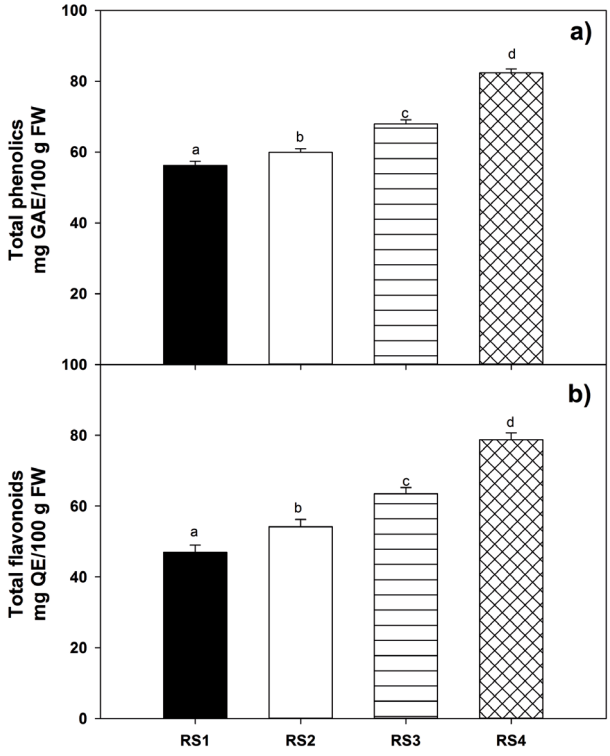

Fig. 2: Changes in total phenolic (a) and flavonoid (b) concentration in pineapple cv. Esmeralda at four ripening stages (RS). Different letters indicate significant differences ($p < 0.05$), $n = 12$.

Antioxidant capacity

Fig. 3 shows the antioxidant capacity of pineapple cv. Esmeralda. DPPH and ABTS inhibition percentage exerted by hydrophilic and lipophilic extracts increased during ripening, and was similar in both assays. Remarkably, antioxidant capacity was mostly due to hydrophilic compounds. In the case of DPPH inhibition, the hydrophilic extracts showed percentages ranging from 20.27 to 38.98%, lower than those reported in Cayenne and Gold pineapples, with 35.87 and 43.1%, respectively (Ayala-Zavala et al., 2010; Montero-Calderon et al., 2010). In contrast, lipophilic extracts showed inhibition rates of around 2%. Other tropical fruits like Ataulfo mango (*Mangifera indica* L.), Maradol papaya (*Carica papaya* L.) and Hass avocado (*Persea americana* Mill.) presented TEAC values of 868, 300 and 270 μm TE/100 g FW, respectively (Corral-Aguayo et al., 2008; Robles-Sanchez et al., 2009), higher than those presented in Esmeralda pineapple at RS3 (14 μm TE/100 g FW).

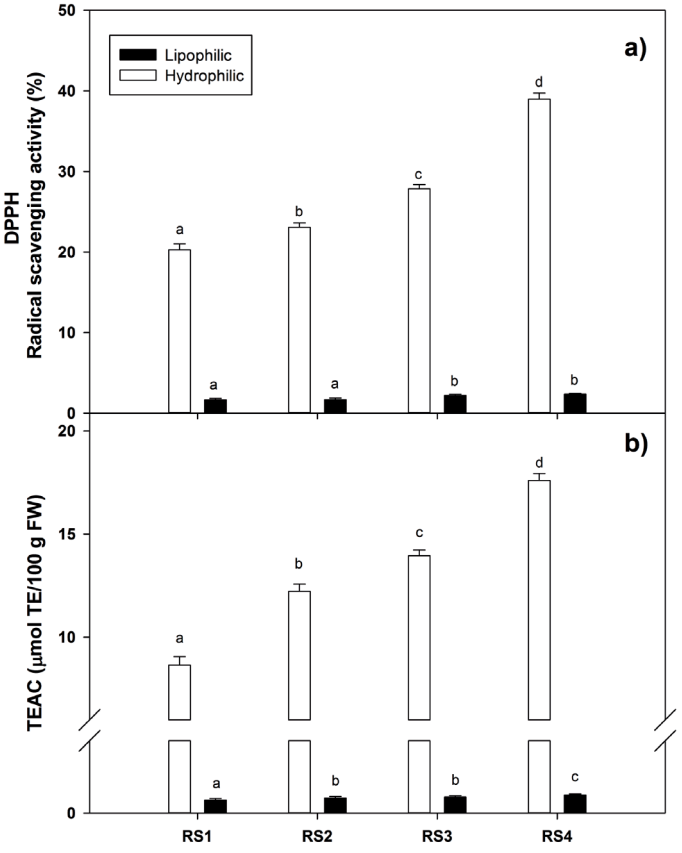

Fig. 3: Antioxidant capacity of hydrophilic and lipophilic extracts of pineapple cv. Esmeralda at four ripening stages (RS). (a) DPPH and (b) TEAC. Different letters indicate significant differences ($p < 0.05$), $n = 12$.

Identification and quantification of phenolic compounds

Tab. 2 shows variations in phenolic content during ripening of pineapple cv. Esmeralda. Gallic acid, catechin, epicatechin, vanillic acid, 2-hydroxycinnamic acid and myricetin were identified. Information about the type and content of phenylpropanoids in pineapple flesh is limited, even more so during ripening. According to the USDA (United States Department of Agriculture, 2014), flavonoids found in highest concentration in pineapple flesh of commercial maturity are quercetin, myricetin and luteolin, of which only myricetin was found in the present study. This may be because

Tab. 2: Phenolic content of pineapple cv Esmeralda at four ripening stages (RS), as detected by HPLC

RT	Compound	RS1	RS2	RS3	RS4
min		mg/g of dry weight (dw)			
13.7	gallic acid	0.9684±0.0011^{d}	0.9548±0.0040^{c}	0.9442±0.0016^{b}	0.7941±0.0011^{a}
19.9	catechin	0.1606±0.0004^{c}	0.1572±0.0010^{b}	0.1780±0.0008^{d}	0.1456±0.0014^{a}
20.8	epicatechin	0.0464±0.0003^{b}	0.0440±0.0003^{a}	1.0700±0.0004^{c}	1.0915±0.0008^{d}
21.9	vanillic acid	0.0117±0.0002^{a}	0.0119±0.0001^{b}	0.0170±0.0003^{c}	0.0289±0.0002^{d}
28.8	2-hydroxycinnamic acid	0.0011±0.0001^{a}	0.0019±0.0001^{c}	0.0014±0.0001^{b}	0.0021±0.0001^{d}
30.5	myricetin	0.0116±0.0000^{d}	0.0025±0.0000^{a}	0.0084±0.0001^{c}	0.0047±0.0001^{b}

RT: retention time, RS: ripening stage. Different superscript letters in the same row indicate significant differences ($p < 0.05$), n = 12.

the type and concentration of these compounds is dependent on many factors, such as variety, environmental conditions, pre- and post-harvest factors and different types of stress (Gonzalez-Aguilar et al., 2010). Phenolic acids are very effective antioxidants due to the carboxyl group that easily ionizes, and to the hydroxyl groups, which allow quick hydrogen atom transfer (Rajan and Muraleedharan, 2017). In contrast, flavonoids like catechin, epicatechin and myricetin are considered good antioxidants because of the number and position of hydroxyl groups. Phenolic compounds also play an important physiological role during fruit ripening. In the early stages of ripening they are found in polymerized form and are a natural part of the defenses of the fruit against pathogen attack (Limem et al., 2008). Moreover, their role as antioxidants is well known, which can protect plant tissues against various types of stress and modulate cellular processes of organisms that consume them.

Some trends on the phenolic profile were notable during pineapple ripening. Gallic acid is the most abundant phenolic at RS1 and RS2. Its concentration slowly decreases (approximately 2.5%) from RS1 to RS3, but it abruptly changed in RS4 (approximately 18%). In contrast, epicatechin concentration increased from RS1 to RS4 (approximately 2.3%). These opposing trends showed that gallic acid is the most representative phenolic compound found in pineapple cv. Esmeralda during earlier stages, but epicatechin is more abundant during later stages. This profile is consistent with other varieties, for example, Du et al. (2016) analyzed the phenolic composition of 17 ripe pineapple varieties grown in China, and found that epicatechin predominates over gallic acid in 15 of them.

The remaining phenolic compounds detected also presented changes in concentration during pineapple ripening. The concentrations of vanillic acid and 2-hydroxycinnamic acid significantly increased, while catechin and myricetin significantly decreased from RS1 to RS4. Nevertheless, the cumulative concentration of phenolic compounds steadily and significantly increased from RS1 to RS4 (Fig. 2).

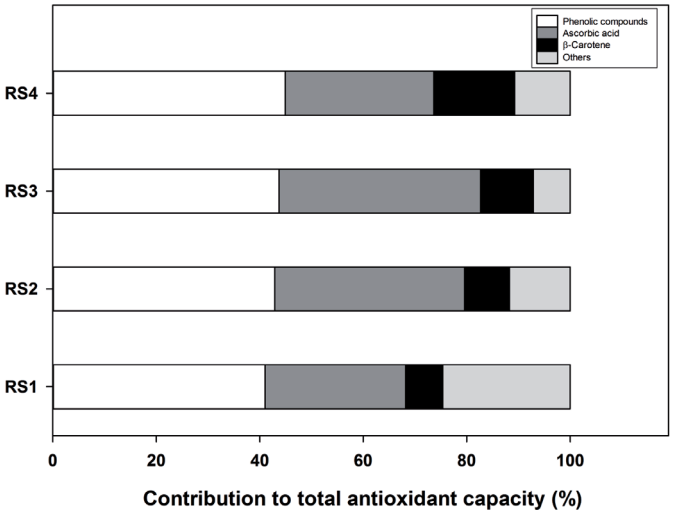

Fig. 4: Contribution of bioactive compounds to total antioxidant capacity of pineapple cv. Esmeralda at four ripening stages (RS). Data was calculated with the DPPH assay, using Equation 1.

Contribution of individual bioactive compounds to total antioxidant capacity

Fig. 4 shows the contribution of individual bioactive compounds to total antioxidant capacity during ripening of pineapple cv. Esmeralda. Phenolic compounds were the most prominent contributors, showing percentages above 40% in all RS. Despite the fact that vitamin C was the bioactive compound with the highest concentration, its greatest contribution to total antioxidant capacity occurred at RS3 (38.93%). β-carotene contributed about 10%. These results are consistent with those reported by Martínez-Flórez et al. (2002) and Escamilla-Jiménez et al. (2009), because even when the concentration of phenolic compounds was lower than the concentration of vitamin C, they exerted greater antioxidant capacity than vitamin C and β-carotene. To the best of our knowledge, no reports are available that evaluate the individual contribution of bioactive compounds to total antioxidant capacity during pineapple ripening. In strawberries (*Fragaria* × *ananassa*), the largest contribution to antioxidant capacity is provided by vitamin C (24%) and phenolic compounds contribute the most to the antioxidant capacity of potatoes (Aaby et al., 2007; Reddivari et al., 2007).

We evaluated the inhibition percentage of DPPH radical of individual phenolic compounds identified. Fig. 5 shows DPPH radical inhibition of different phenolic compounds in increasing concentration. It is clear that gallic acid was the most effective antioxidant of phenolic nature, followed by epicatechin, myricetin and catechin. In contrast, vanillic acid and 2-hydroxycinnamic acid had the lowest values. Gallic acid was also found at the highest concentration and contributed a higher percentage to total antioxidant capacity, however, its concentration remained constant during the early stages of ripening and decreased at RS4, even when the content of phenolic compounds and their contribution to antioxidant capacity continued to increase.

Fig. 6 shows the basic structure and substituent groups found in the identified phenolic compounds. The high antioxidant potential of gallic acid is well known, which is attributed mostly to its three available hydroxyl groups that can easily donate a hydrogen atom

(Rajan and Muraleedharan, 2017). The low DPPH inhibition exerted by vanillic acid and 2-hydroxycinnamic acid could be due to their structure that contains a single hydroxyl group, which may interact with the methoxy or carbonyl group, respectively, preventing hydrogen atom donation.

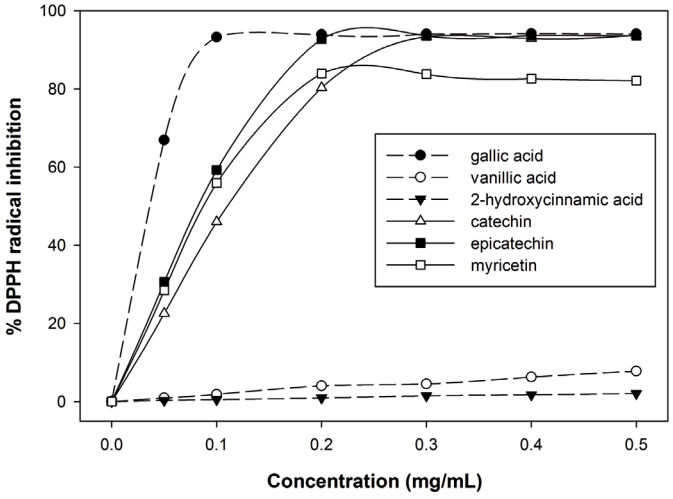

Fig. 5: DPPH radical inhibition percentage of phenolic compounds identified in pineapple cv. Esmeralda.

Enzyme activity

Fig. 7 shows PAL (7a) and POD (7b) activity in pineapple cv. Esmeralda at different RS. PAL activity increased gradually and significantly from RS1 to RS4. Our results are in accordance with those reported for strawberries, where PAL activity increases during ripening (Song et al., 2015). Increased PAL activity has also been reported in response to different types of stress, such as treatments with ozone (Duarte-Sierra et al., 2016) and others. PAL is a key enzyme of the phenylpropanoid pathway, which reacts against various kinds of stress (Ganapathy et al., 2016).

The increase in PAL activity during ripening showed a good correlation with total phenolics (r = 0.98) and total flavonoids (r = 0.98). Similar results have been found during ripening of Golden Delicious apple (Blankenship and Unrath, 1988) and in fresh-cut pineapple cv. Josapine, PAL activity has been recently associated with increased antioxidant capacity (Yeoh and Ali, 2017).

Phenolic compounds are considered substrates of PPO and POD during browning reactions (Min et al., 2017). We detected no PPO activity at any RS, which is in agreement with the results of Montero-Calderon et al. (2010) in Gold pineapples. This suggests synthesis of phenolics is highly favored over their degradation.

POD activity increased gradually and significantly from RS1 to RS4. Increased POD activity during ripening significantly correlated with concentration of total phenolics (r = 0.83) and total flavonoids (r = 0.88). An increase in POD activity occurs in various conditions; in other fruits, vegetables and plant tissues, increased POD activity takes place when the tissue in question has been stressed (Kaya

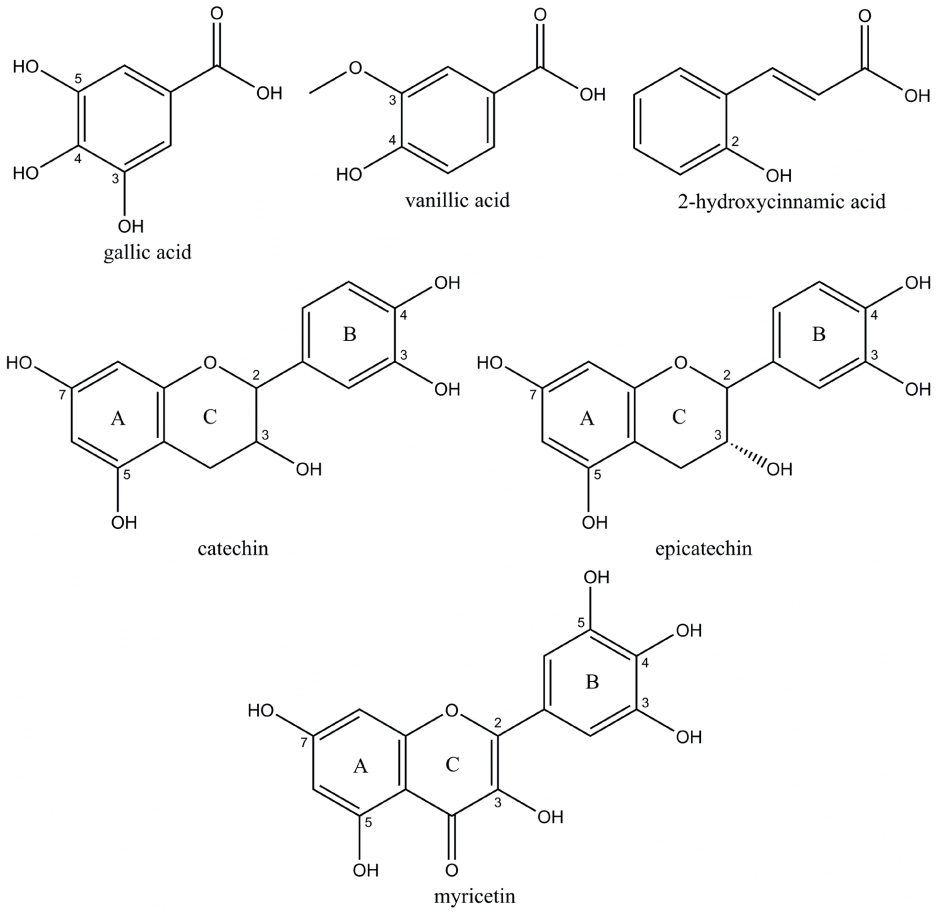

Fig. 6: Molecular structure and substituent position of identified phenolics in pineapple cv. Esmeralda. Gallic acid, vanillic acid and 2-hydroxycinnamic acid are simple phenolic acids with one (vanillic acid and 2-hydroxycinnamic acid) or three (gallic acid) hydroxyl groups. Catechin, epicatechin and myricetin are flavonoids of more complex structure that have five (catechin and epicatechin) or six (myricetin) hydroxyl groups.

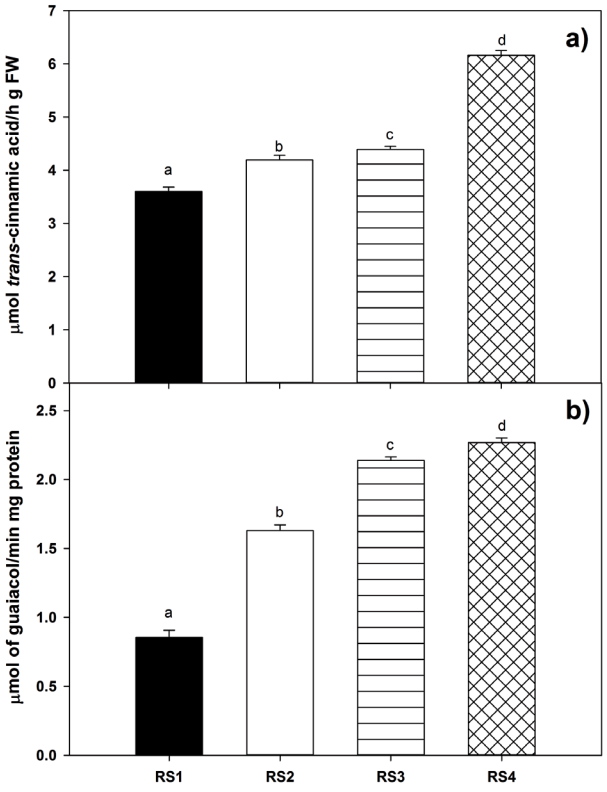

Fig. 7: Enzyme activity of phenylalanine ammonia lyase, PAL (a) and peroxidase, POD (b) from pineapple cv. Esmeralda at four ripening stages (RS). Different letters indicate significant differences ($p < 0.05$), $n = 12$.

and Ashraf, 2015). This may result in protection against oxidative stress and slowed cell degradation by making phenolic compounds available substrates for POD (Raimbault et al., 2011).

Conclusions

Ripening stage significantly influenced most measured parameters, except for pH. Bioactive content and antioxidant capacity increased throughout the ripening process. Among the identified phenolic compounds, gallic acid, catechin and epicatechin had the highest concentration. In addition, our study showed that although concentration of phenolics was less than vitamin C, they contributed more to total antioxidant capacity. PAL and POD activities increased with fruit ripening, showing positive correlations with phenolic compound concentration. Esmeralda pineapple is a good source of bioactive compounds with potential health effects, but their concentrations vary with ripening stage.

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