Revista Ciência Agronômica, v. 55, e20218169, 2024
Centro de Ciências Agrárias - Universidade Federal do Ceará, Fortaleza, CE
www.ccarevista.ufc.br

Scientific Article
ISSN 1806-6690

Biochemical changes and peroxidase activity during development of acerola Okinawa[1]

Christine Maria Carneiro Maranhão Ribeiro[2*], Tatyana Patrício de Albuquerque Sousa[2], Marco Túlio José de Barros Ribeiro[3], Larissa Raphaela Gonçalves Farias[4], Heinz Johann Holschuh[5], Silvanda de Melo Silva[6], Taliana Kênia Alencar Bezerra[7], Maria Inês Sucupira Maciel[8]

ABSTRACT - In this study, chemical changes during the development of acerola, cultivar Okinawa, were evaluated. The biochemical alterations during the development of the acerola cultivar Okinawa were evaluated, to determine the peroxidase activity (POD) and its thermal stability. Peroxidase (POD) has been considered the main enzyme responsible for the quality deterioration of fruits. Additionally, characteristics evaluated in this study can be important postharvest quality criteria for fruit processing, screening and breeding. Changes in physiological development were evaluated by the determination of moisture content, ascorbic acid, ashes, total and reducing sugars, pectin, chlorophyll a and b, amounts of anthocyanins, flavonols and total carotenoids. These biochemical determinations may serve as a basis for further improvement of techniques for predicting and modeling the optimal harvesting time. The values of chlorophylls, anthocyanins, flavonols, and carotenoids were higher in the peel rather than in the pulp ($p < 0.05$). Acerola presents high levels of ascorbic acid. The development of acerola (from opening of flowers to maturation of fruits) was of 25 days. The increase of the values for total and reducing sugars, anthocyanins, carotenoids, the solubilization of pectic acid and degradation of chlorophyll suggested that physiological maturation and harvest day occurred on days 19 and 23 after anthesis, respectively. The specific activity of POD was higher in the green stage. Maximum POD activity was observed at pH 6.5 and 45 ºC. Total inactivation was not achieved, suggesting the presence of isoenzymes with high thermal resistance. A loss of 50% of activity occurred after 4 minutes of treatment at 80 °C.

Key words: Bioactive compounds. Enzyme activity. Thermal Stability.

DOI: 10.5935/1806-6690.20240008
Editor-in-Chief: Prof. Alek Sandro Dutra - alekdutra@ufc.br
*Author for correspondence
Received for publication 19/08/2021; approved on 25/07/2023
[1]Part of the of two Master's Dissertation of the first authors presented Federal University of Paraíba (UFPB). Unfunded research
[2]Department of Food Engineering, Postgraduate program in Food science and technology, Federal University of Paraíba (UFPB), João Pessoa-PB, Brazil, tina_maranhao@yahoo.com.br (ORCID ID 0000-0003-0472-3954); tatypatricicoutinho@gmail.com (ORCID ID 0000-0003-3697-8417)
[3]Federal University of Pernambuco (UFPE), Recife-PE, Brazil, marcot_ribeiro@yahoo.com (ORCID ID 0000-0003-2630-1760)
[4]Department of Food Engineering, Postgraduate program in Food science and Technology, Federal University of Paraíba (UFPB), João Pessoa-PB, Brazil, larissargfarias@hotmail.com (ORCID ID 0000-0002-0261-9718)
[5]Department of Food Engineering, Postgraduate program in Food science and Technology, Federal University of Paraíba (UFPB), João Pessoa-PB, Brazil, hj.holschuh@ct.ufpb.br (ORCID ID 0000-0001-6366-9316)
[6]Department of Agronomy, Postgraduate program in Food science and Technology, Federal University of Paraíba (UFPB), Campus II, Areia-PB, Brazil, silvasil@cca.ufpb.br (ORCID ID 0000-0003-2106-6458)
[7]Department of Food Engineering, Postgraduate program in Food science and Technology, Federal University of Paraíba (UFPB), João Pessoa-PB, Brazil, taliana.kenia@hotmail.com (ORCID ID 0000-0002-4388-4024)
[8]Department of Consumer Sciences, Postgraduate program in Food science and Technology. Rural Federal University of Pernambuco (UFRPE), Recife-PE, Brazil, m.ines@dcd.ufrpe.br (ORCID ID 0000-0002-8910-2833)

INTRODUCTION

Acerola, or Cherry Antilles, (*Malpighia emarginata* DC) is a fruit that originated in the Antilles, North, South and Central America. The plant was introduced in Brazil in the 50s, and is well-adapted to the climate and soil (LEFFA *et al.*, 2015) these pathological conditions are associated with elevated markers of oxidative stress. Acerola fruit contains high levels of vitamin C and rutin and exhibits corresponding antioxidant properties. In this study, we investigated the antioxidant effects of acerola juice in different stages of maturity (unripe, ripe, and industrial. The demanding market has pushed the discovery of new cultivar of acerola with enhanced resistance to diseases, pests, and mechanical damage, as well as with enhanced color, size, and improved chemical composition.

The economic potential of this fruit is based on its high ascorbic acid content, which can vary from 342.47 to 2,649.72 mg 100 g^{-1} (MARANHÃO RIBEIRO *et al.*, 2018) and the presence of several other bioactive compounds including amino acids (AA), β-carotenoids (CA), phenolic compounds (PC) and minerals (XU *et al.*, 2020). The levels of sugar, acids, anthocyanins, total phenolics, ascorbic acid, and antioxidant activity of a species is strongly affected by cultivar, season and fruit maturation at harvest (BRAVO *et al.*, 2015). For instance, vitamin C content and composition of phenolic compounds also depend upon the maturation stage (XU *et al*., 2020). The maturation stages are characterized by color and quality changes. Sugars and acids have an influence on the flavor and aroma of fruits. Sugars, especially sucrose, glucose, and fructose, are responsible for fruit sweetness, while organic acids, particularly citrate and malate, determine fruit acidity (LI *et al*., 2020).

Several physical, chemical and biochemical changes take place during fruit development. Their knowledge is important for post-harvest technology and for industrial processing procedures of plant products. One factor that influences the quality of the fruit is the harvesting time. The ideal harvest point depends on the destination for fresh consumption or agro-industrialization. So it is fundamental to determine the maturity index which includes physical or chemical measures that undergo noticeable changes during maturation to ensure the production of fruit with quality characteristics and appropriate behavior during storage (SILVA *et al*., 2016).

These indicators are used to determine the optimal harvesting time to obtain improved features for fresh consumption. Fruit ripening is a highly coordinated, genetically programmed, and irreversible phenomenon involving physiological, biochemical, and organoleptic changes that lead to the development of a soft edible ripe fruit with desirable quality attributes (EL-SHARKAWY *et al*., 2016).

Peroxidases (EC.1.11.1, POD) are implicated in several vital processes as part of cell wall metabolism, lignification, reactive oxygen species (ROS) metabolism, fruit growth and maturation. The peroxidase activity (POD) can vary with stage of maturity of the fruit. It can also generate a major problem during harvesting, transport, storage and processing of fruits by causing change in color, taste and loss of nutritional value, making them unacceptable for consumption (SINGH *et al*., 2018).

Few studies have assessed biochemical changes in acerola through the evaluation of fruit development from flowering to ripening (AWAD *et al*., 2017; OLIVEIRA *et al*., 2012). Given the significant levels of nutritional and economic interest in the acerola fruit, the objective of this study was to evaluate the biochemical changes during the development of acerola, Okinawa cultivar, to determine the peroxidase activity and thermal stability at three stages of maturation of this fruit, in addition to determining the optimum pH and temperature for this enzyme.

MATERIALS AND METHODS

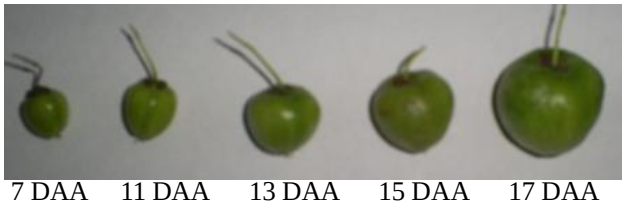
Plant material

Acerola (*Malphigia emarginata* DC, Okinawa cultivar) fruit was obtained from the Morimtzu farm in Alhandra, Paraíba, Brazil, coordinates: 7° 26’ 22” South and 34 °54’ 49” West. Acerola was harvested from one hundred and fifty trees aged 14. The place has tropical rainy weather with dry summers. The mean precipitation is 1,600 mm and temperatures vary from 18 ºC to 30 ºC. The planting area has the following characteristics: The soil is sandy, located in the West position, in an area 100 hectares. The orchards of acerola were irrigated by sprinkling. The seedling was done by grafting, using as a rootstock to cultivar ‘Flor branca”. The production of the acerola seedling of the Okinawa Cultivar was by the grafting method.

Sampling

Freshly opened flowers were tagged and, after fruit development, approximately seven days after the anthesis, and in the period of 2 and 4 days, batches of 300 g of fruit were harvested with the objective of evaluating fruit quality during development (fig 1). Fruit was crushed separately in a food processor (Philips Walita, Mega Master Pro RI 3173) according to their different maturation stages and stored at -18 ºC prior to chemical analyses.

Figure 1 - Acerola (*Malpighia emarginata* DC) development scale of cultivar Okinawa



7 DAA 11 DAA 13 DAA 15 DAA 17 DAA

DAA: days after anthesis

Biochemical Analysis

Moisture, protein and ash content were analyzed according to the Association of Official Analytical Chemists (2012). The content of total and reducing sugars were determined by the colorimetric method Nelson (1960). Pectin was measured according to the method described by Rangana (1977). Ascorbic acid levels/ concentrations were analyzed according to the Association of Official Analytical Chemists (2012). The ascorbic acid content was quantified using 2,6-dichlorophenol indophenol (DCPIP) with modification using oxalic acid solution as solvent, replacing metaphosphoric acid.

The analysis of carotenoids, chlorophyll, anthocyanins and flavonols were performed on the same day of harvest to avoid analytical errors. They were performed in the acerola peel and pulp. The peel was removed manually with the aid of a stainless steel knife, while to obtain the pulp, a Walita food processor was used.

For the extraction of chlorophyll and carotenoids from the peel and pulp, 1 g of peel/pulp was weighed and macerated with the aid of a mortar and a pestle in 7.0 mL of 80% (v/v) acetone, in a dark room. The extract was filtered on qualitative filter paper (J prolab), with a porosity of 14 $L.s^{-1}$ m^{-2} and a diameter of 11.5 cm, to volumetric flasks protected against light, involved with aluminum foil. The filter was washed twice with 7.0 mL of 80% acetone and the volume made up to 25 mL. The upper extract was transferred to a 25 mL volumetric flask, completing the volume with acetone.

Carotenoid and chlorophyll contents were determined according to the proposed equation by Lichtenthaler and Buschmann (2001) and the method reported by Rodrigues-Amaya (1999) (Equations 1, 2 and 3).

$$(Totalcarotenoids^{-1})[1000 \times A470 - (1.82Ca - 85.02 \times Cb)]/198 \quad (1)$$

$$(Chlorophyll'a^{-1})12.25 \times A663 - 2.79 \times A646 \quad (2)$$

$$(Chlorophyll'b^{-1})21.5 \times A646 - 5.10 \times A663 \quad (3)$$

*A470 = carotenoid concentration at 470 nm; Ca = chlorophyll a concentration; Cb = chlorophyll b concentration b; A663 = chlorophyll a concentration at 663 nm; A646 = chlorophyll b concentration at 646.

For the extraction of anthocyanins and flavonoids, 1.0 g of peel and pulp were weighed and then stored with 3.0 mL of the extracting solution 95% (v/v) of ethanol - HCl (1.5 N) in the proportion 85:15 for 12 h at 4 °C. The peel was macerated with the aid of a mortar and a pestle in 7.0 mL of the extraction solution, in a dark room. The extract was filtered on qualitative filter paper (J prolab), with a porosity of 14 $L.s^{-1}$-m^{-2} and a diameter of 11.5 cm, to volumetric flasks protected against light, involved with aluminum foil. Then, the volume was made up to 25 mL and it was left for two hours. All physicochemical and chemical analyzes were performed in three replicates.

Total contents of anthocyanin and flavonols were determined in the fruit's peel and pulp according to the equation proposed by Murray and Hackett (1991) and methodology reported by Lees and Francis (1972) (Equations 4 and 5).

$$(Anthocyanins^{-1})A532 - 0.24A653 \quad (4)$$

$$(Flavonols^{-1})A374 - 0.24A653 \quad (5)$$

A532 = anthocyanin concentration at 532 nm. A374 = Flavonols concentration at 374 nm.

2.4 Preparation of samples and enzyme extraction.

Biochemical evaluation was carried out in three stages of maturation with the following characteristics: 1 - Totally green fruit (17 DAA) – turgid and firm, with smooth and shiny surface; 2 – Green-orange fruit (21 DAA) – with 50% green and 50% orange color, turgid, slightly firm, with smooth and shiny surface and 3 - Red fruit (25 DAA) - slightly turgid, with a complete loss of firmness, liquid pulp, and smooth and shiny surface (Figure 2). Fruit was manually harvested, and 500 g of fruit of uniform size and without apparent defects (spots, diseases and damage) were selected for each of the stages of maturity.

Enzyme extraction was performed using the methods proposed by Khan and Robinson (1994). Fruit was washed in distilled water, dried separately at room temperature and then macerated using a mortar and a pestle in the presence of 0.2 M sodium phosphate buffer with a pH of 8.0, containing EDTA, $CaCl_2$ and

PEG 10,000 at final concentrations of 0.01 M, 0.2 M and 2% (w/v), respectively. Throughout this experimental stage, plant material was retained in an ice bath at 4 °C. Extracts were centrifuged at 5,000 rpm for 10 min at 4 °C, and the supernatant was kept at -20 °C until analysed.

Quantification of soluble proteins

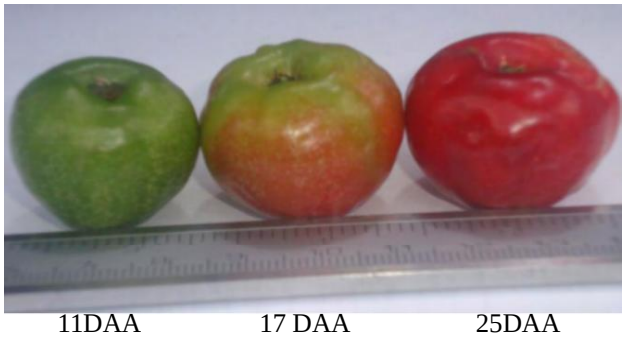
Soluble proteins were quantified according to the method described by Lowry *et al*. (1951). The samples were incubated at room temperature for 10 minutes in the presence of a cupro-alkaline reagent. Then, 25 µL samples were added to a dilute solution of water plus Folin Phenol reagent for 30 minutes. The absorbance was read at 660 nm. The protein concentration was determined using a standard curve for bovine serum albumin at concentrations ranging from 5 to 200 µg mL^{-1}.

Determination of L-acorbic acid and Peroxidase activity (POD)

The determination of ascorbic acid was quantified according to the methodology proposed by the Association of Official Analytical Chemists (2012). In preliminary analyzes, it was verified that the peroxidase did not show enzymatic activity through the adopted methodology, which measures the increase in absorbance of the reactive mixture constituted of guaiacol and hydrogen peroxide in a 0.05M phosphate-citrate buffer pH 6.0. Then, the L-ascorbic acid elimination methodology was adopted, concentrating the enzymatic extract through lyophilization (Lyophilizer brand Terroni LS3000) followed by dialysis against distilled H_2O for 24 hours with two changes and, then, two more changes using 0.05M phosphate buffer pH 6 .0 for another 24 hours. The pilot test was carried out using acerola in the mature, fresh and green stages to verify the efficiency of the procedure.

POD assays were performed as described by Khan and Robinson (1994). Enzyme activity was measured in a reaction system containing H_2O_2 and guaiacol as a substrate. The reaction mixture consisted of 1.5 mL of 1% (v/v) guaiacol and 0.4 mL of 3% (v/v) H_2O_2 in a 0.05 M phosphate-citrate buffer at pH 6.0 and incubated at 25°C. Next, 0.2 mL of enzyme extract was added, and readings at 470 nm, which were monitored for 1 minute of reaction at 25 °C.

Figure 2 - Fruit of the acerola tree in three stages of maturation



11DAA 17 DAA 25DAA

Determination of optimal temperature and pH

The influence of temperature and pH on peroxidase activity was determined for the temperature range from 40 to 70 °C at intervals of 5 °C and for the pH range from 4.0 to 7.0, with intervals of 0.5 pH units. Temperatures were controlled using a circulating water bath.

Thermal stability and inactivation

Enzyme extract samples in test tubes (selected to be equal in weight, volume and size) were incubated in a water bath at temperatures of 30, 50 and 70 °C for 0, 12 and 24 hours. After thermal treatment, samples were cooled in an ice bath for at least 30 minutes, and the residual activity was measured.

The residual activity of the POD enzyme extract was determined after the following incubation times and temperatures: 70, 80 and 90 °C for 0, 2 and 4 minutes, according to the methodology described for the thermal stability determination.

Recovery of enzyme activity

After enzyme extracts remained at room temperature for 24 hours, the thermal inactivation activity of the samples was reassessed to determine the extent of recovery of activity.

Statistical analysis

The results of the characterization of the biochemical composition were submitted to regression analysis. They were made using the *F* test to determine their significance at 5% probability. The models were evaluated in terms of *F*-ratio and R^2 coefficient. All statistical analyses were performed in the *R* environment using the 2.13.0 version of the free software available at http://cran.r-project.org/.

To determine the optimum temperature and pH, a rotational central composite design (RCCD) was carried out, 2^2 composed by 4 factorial points (levels ± 1), 3 central points (level 0), and 4 axial points (levels ± α), totalling 11 tests. The thermal stability and thermal inactivation were designed a 22 full factorial design, with a central point (Table 2 and 3). Data were analyzed by ANOVA ($p < 0.05$) and, when there were significant differences between the treatments, response surfaces were generated to find the optimal region of pH and temperature.

Table 1 - Values used in the Central Composite Rotational Design (CCRD) to determine the optimum temperature and pH

Variables	+α	Level (1)	Level (0)	Level (-1)	-α
Temperature (°C)	70	65	55	45	40
pH	7.0	6.5	5.5	4.5	4.0

Table 2 - Values used in full factorial design for thermal stability

Variables	Level (1)	Level (0)	Level (-1)
Temperature (° C)	70	50	30
Time (H)	24	12	0

Table 3 - Values used in full factorial design for thermal inactivation

Variables	Level (1)	Level (0)	Level (-1)
Temperature (° C)	90	80	70
Time (min)	4	2	0

RESULTS AND DISCUSSION

Biochemical Analysis

Ascorbic acid content decreased during fruit development (Figure 3A). The regression analysis on the ascorbic acid data showed a quadratic representation. The F cal. was larger than the F tab. revealing that the model fits well with the experimental data. This behavior was in agreement with those determined by Xu *et al.* (2020) in acerola during development. This decrease is due to ascorbic acid oxidation to dehydroascorbic acid through the action of ascorbate oxidase.

The total and reducing sugar levels increased during fruit development, presenting a cubic curve while the total sugar showed a quadratic equation with R^2 0.88 and 0.86, respectively (Figure 3B). The F cal > Ftab showed value of $p \leq 0.05$, indicating that the proposed model was significant. Li *et al.* (2020) sugars and organic acids determine fruit organoleptic quality and drastically change during fruit maturation. This study examined enzymes involved in the metabolism of sugars and organic acids during the three maturation phases (green, pink and blueattributed this increased sugar content during development to the increased levels of fructose, glucose, and sucrose during maturation stages and probably due to the degradation of the starch during the ripening process.

The moisture content increased during the development of acerola (Figure 3C). Moisture content presented a cubic behavior. The R^2 was 0.84 with a value of $p \leq 0.05$. The F test indicated that the model was significant. Patil and Shanmugasundaram (2015) explain that this gain is due to the increase of the sugar content in the pulp as a result of the hydrolysis of the starch to sugar.

The ash result presented a cubic comparison, with an explained variation (R^2) of 0.89. The ash contents declined during fruit development and reached values of 0.24% on day 25 after anthesis (Figure 3D). The F cal. was larger than the F tab. revealing that the model fits well with the experimental data.

Protein content decreased up to day 15 after anthesis and increased and decreased up to day 25 after anthesis (Figure 3E). The decreased protein content during fruit development was probably the result of the relative increase in fruit volume and increased transcription processes linked to the synthesis of new enzymes involved in the maturation process (LI *et al*., 2020). The protein content showed a cubic curve behavior. The value of explained variance (R^2) was 0.76 and F cal. was higher than the F tab., indicating that the proposed model was significant. Amino acids are well-known precursors of volatile compounds formed during maturation. Although this decreased protein content would not have a nutritional impact, it might affect sensorial fruit characteristics due to the formation of volatiles with an impact in the aroma. The concentration increased between day 11 and 21, after anthesis and declined afterward (Figure 3E).

A significant increase in anthocyanins content was observed 25 days after anthesis in both peel and pulp (62.72 and 5.16 µg g^{-1}) (Figures 3F and 3G). A polynomial fit was not possible using the anthocyanins

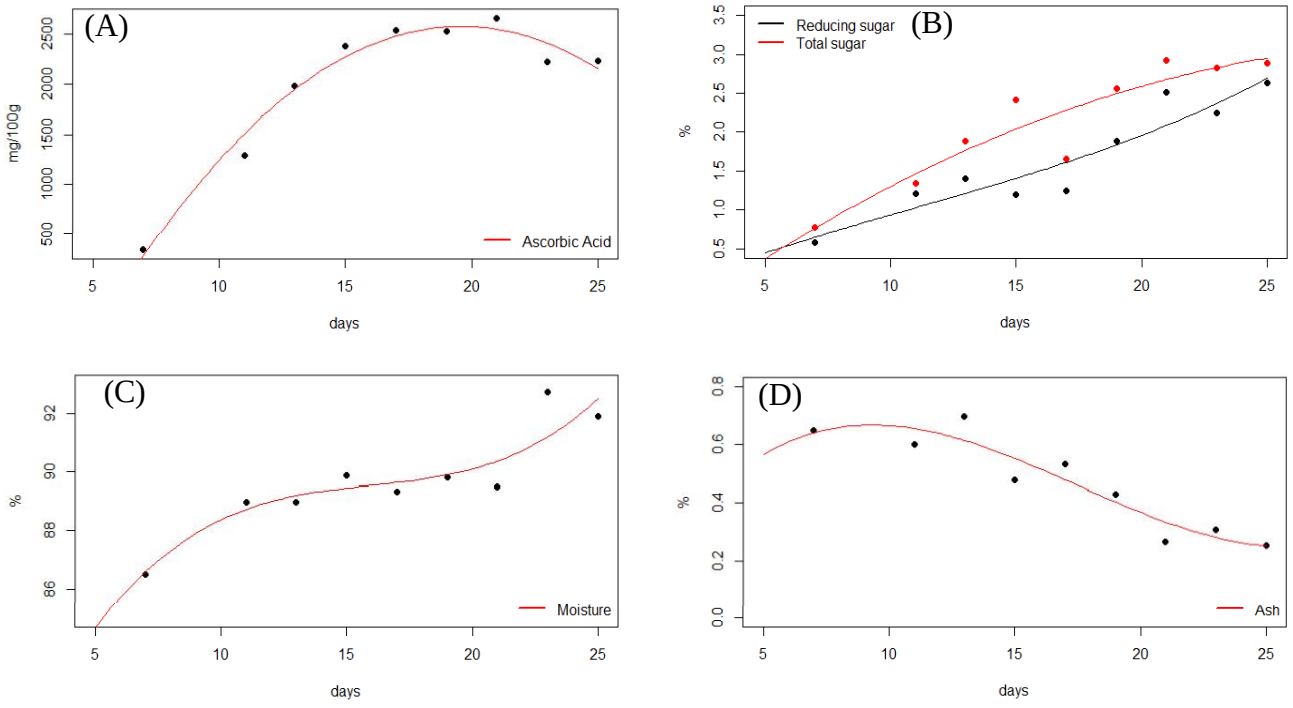
data from acerola peel and pulp because the data behaved exponentially. The content of anthocyanins in the pulp was lower to that found by Oliveira *et al.*(2012) when studying the development of the acerola clone BRS 235 (0.45 to **6.**49 mg 100g^{-1} of total anthocyanins).

The flavonols content decreased until day 17 and then increased along with the rise of anthocyanins (Figures 3F e 3G). The regression analysis in the flavonols data showed a cubic representation. The F cal. was higher than the F tab. This indicates that the proposed model is significant. Gordilho *et al.* (2015) explain that individual flavonoids cause more intense and stable color of anthocyanins than the pigments alone. The synthesis of flavonols occurs before the synthesis of anthocyanins. A significantly higher content of flavonols in the peel was found 25 days after anthesis (25.92 to 2.85 µg g^{-1}).

The levels of carotenoids and clorophyll decreased during development, but at the end of maturation there was an accumulation of carotenoids (Figure 3H, 3I). The carotenoid data in peel and pulp showed cubic behaviors, with an explained variation of R^2 of 0.84 and 0.90, respectively. The F cal. values were higher than the F tab., therefore it can be concluded that the models fit well to the experimental data. Solovchenko, Avertcheva and Merzlyak (2006) explain that analysis of this relationship revealed a strong dependence between fractions of carotenoids accumulated and those of chlorophylls degraded during ripening, regardless of harvest date and pigment content of the fruit. The high carotenoids content in the early development of acerola in the peel and pulp (19.79 to 14.2 µg g^{-1}) is probably due to high content of lutein.

It is observed in Figure 3I that there was a correlation between the average values of chlorophylls a and peel, which decreased as the fruit development progressed. The chlorophylls a and b data in peel and pulp showed cubic behaviors, with an explained variation of R^2 of 0.82 to 0.97. The degradation of chlorophylls involves the interaction of two enzymes, “pheophorbide a oxygenase” (PaO) and “red chlorophyll catabolite reductase (RCCR); PaO seems to be a key enzyme in the catabolism of chlorophylls, and its activity is only detected during plant senescence (HÖRTENSTEINER, 2013). The findings obtained in this work give grounds to believe that the chlorophylls content could serve as an internal marker of the fruit’s physiological state, indicative of the rate of ripening.

Figure 3 - Regression model for : Ascorbic acid expressed in mg 100g^{-1} (A). Reducing sugar and total sugar expressed percentage (B). Moisture expressed percentage (C). Ash expressed percentage (D). Protein and calcium pectate expressed percentage (E). Anthocyanin and Flavonols in the peel expressed in µg g^{-1} (F). Anthocyanin and flavonols in the pulp expressed in µg g^{-1} (G). Total Carotenoids in the peel and pulp expressed in µg g^{-1} (H). Chlorophyll a and b in the peel and pulp expressed in µg g^{-1} (I)



Continuation Figue 3

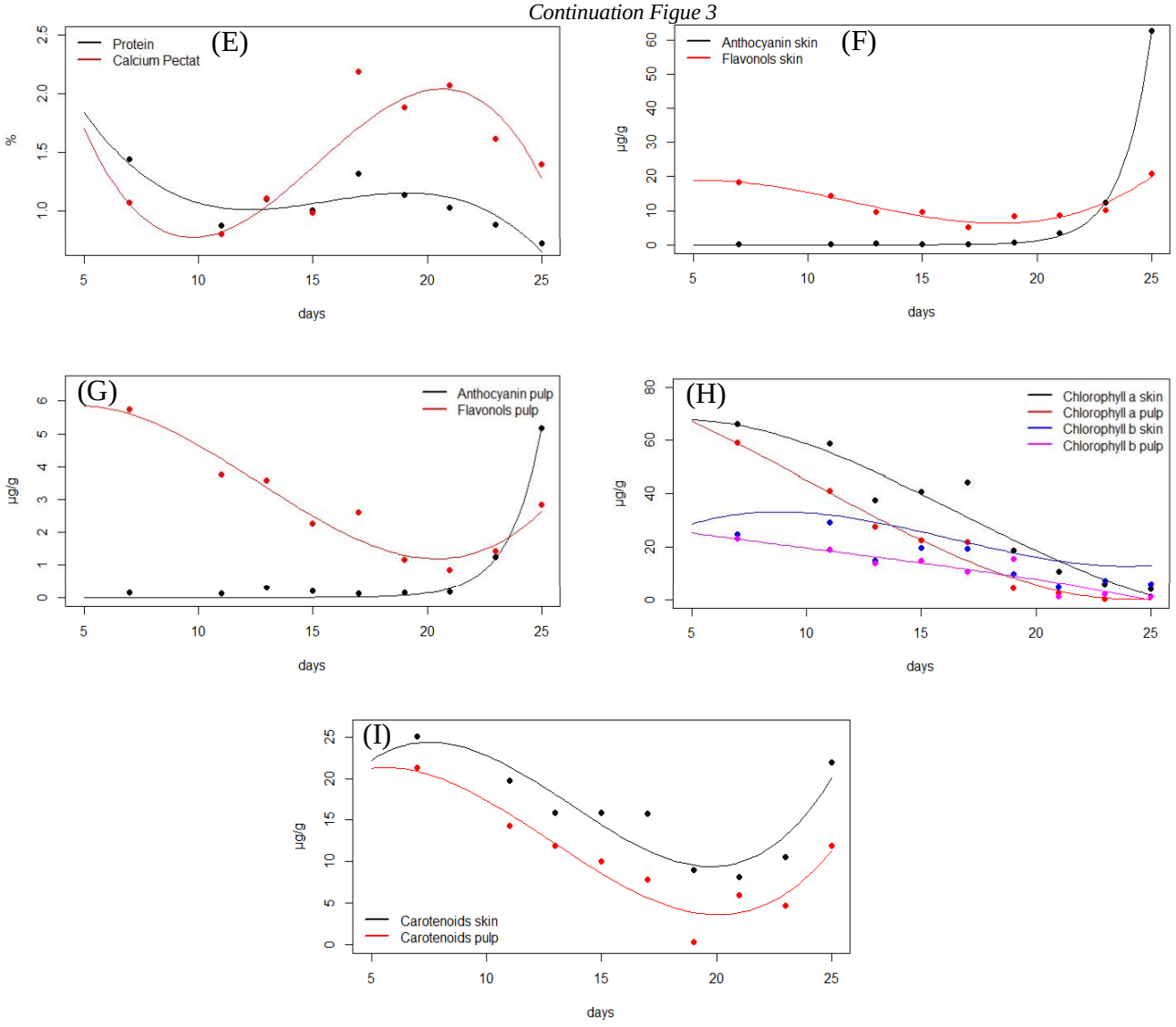


Table 4 - Regression equations (for the coded variables) and statistical parameters of the models for the chemical properties of acerola

Equations	R^2
$Ascorbicacid = -14.56x^2 + 570.3x - 3009.7$	
$totalsugars = -0.003x^2 + 0.24x + 0.75$	0.88
$\text{Re}\,ducingsugars = 0.0001x^3 + 0.0001x^2 + 0.13x + 0.15$	0.86
$Moisture = 0.002x^3 - 0.14x^2 + 2.35x + 76.01$	0.84
$Ash = 0.0001x^3 - 0.009x^2 + 0.13x + 0.11$	0.89
$\text{Pr}\,otein = -0.0008x^3 - 0.042x^2 - 0.63x + 4.04$	0.76
Anthocyanins peel = y~1.40e 0.7*exp (7.96e *01X)	
Anthocyanins pulp = y~6.83e 0.8*exp (7.25e *01X)	
$Flavonolspeel = -0.001x^3 - 0.43x^2 + 3.65x + 9.73$	0.91
$Flavonolspulp = 0.002x^3 - 0.09x^2 + 0.72x + 4.22$	0.93

Continuation Table 4

$Carotenoidspeel = 0.001x^3 - 0.672x^2 + 7.44x + 0.21$	0.84
$Carotenoidspulp = -0.001x^3 - 0.45x^2 + 3.93x + 11.$	0.90
$Chlorophyllapeel = -0.009x^3 - 0.48x^2 + 3.72x + 60.05$	0.92
$Chlorophyllbpeel = -0.012x^3 - 0.59x^2 + 7.65x + 3.58$	0.82
$Chlorophyllapulp = -0.008x^3 - 0.25x^2 - 1.97x + 82.52$	0.97
$Chlorophyllbpulp = -0.0001x^3 - 0.002x^2 - 1.40x + 31.66$	0.85

3.2 Determination of L-acorbic acid and Peroxidase activity

Table 5 presents the results of the pilot test regarding the elimination of L-ascorbic acid and the determination of the enzymatic activity (U) of POD verified in three stages of acerola maturation. It was found that the L-ascorbic acid content was significantly higher ($p < 0.05$) during the green stage, followed by the mature stage, as well as in the crude extract and after 24 hours. After dialysis, the highest residual content was during the mature stage. Enzymatic activity (U) of POD was only measured after dialysis and a significant decrease in L-ascorbic acid, due to its interference in the expression of the enzyme.

The enzyme activity in acerola was evaluated during three stages of ripeness as described earlier. Enzyme activity in the green stage was significantly higher ($p < 0.05$) than in the other stages (Table 6). Awad *et al.* (2017) explain that the increased POD activity in the early stages of fruit development can be explained by its metabolic function of protecting plant against pathogens. The maturation stage with the highest protein value was stage two (Orange Green), with a value of 8.5 mg per $cm^{3.}$ The specific activity of peroxidase was higher in the green stage, with 24.6 U mg^{-1} of protein, and this difference between stages was significant ($p < 0.05$).

Determination of optimal temperature and pH

Figure 4 shows that peroxidase from acerola had a maximum activity level in phosphate buffer with a pH of 6.5 and at a temperature of 45 °C. These findings are similar to those obtained by Amiour and Hambaba (2016), who reported that peroxidase from Ghars showed maximum activity in phosphate buffer with pH of 6.2. While the optimum temperature was close to that found by Gong *et al*. (2015), 50 ºC in chestnuts.

Thermal stability

Figure 5 shows that the maximum peroxidase activity of acerola occurred at a high temperature (T = 70 °C) and minimum time (< 1 hour) and a severe loss in time of 24 hours. The peroxidase activity was stable when subjected to 30 °C at all treatment times up to 24 hours and still had values close to 50 °C at the time-point of 12 hours. This stability behavior is close to thermostability of Ficus carica latex peroxidase which was stable from 30 °C up to 50 °C (ELSAYED *et al.*, 2018).

Thermal inactivation

Figure 6 shows that the peroxidase activity of acerola decreased with the time of exposure to the thermal treatment and with increasing temperature. At 70 °C and 4 minutes, there was a loss of peroxidase activity of approximately 20%. Peroxidase activity of acerola was even lower at 80 °C for 2 minutes compared with 70 ºC for 4 minutes, respectively. A residual POD activity of 50% was achieved at 80 °C for 4 minutes. There was a huge loss of activity at higher temperatures (T = 90 °C) and at the maximum time (4 minutes). Tao *et al*. (2018) studied peroxidase activity in jackfruit, and found that enzyme activity decreased with the time of exposure to the thermal treatment and with increasing temperature. The highest level of enzymatic inactivation was at a temperature of 90 °C. The authors, however, observed that the heat treatments used were not sufficient for the complete inactivation of the enzyme. This complex behavior may be related to the presence of isoenzymes with different heat stability or the protection of protein and sugar in the crude extract.

Recovery of enzyme activity

As seen in Figure 7, the concentrated enzymatic extracts showed some degree of regeneration in the thermal inactivation study. At 90 °C, there was an increase in, or a recovery of, enzyme activity in all samples. At 80 °C, there was a decrease of peroxidase activity, showing a slight recovery of the treatments in the shortest time and losses at the times of 3 to 4 minutes. At 70 °C, there was no recovery of the treated POD activity at any of the treatment times. Siguemoto *et al.* (2018) also reported over-activation of POD from apple juice after microwave heating and conventional heating. The authors

demonstrated that regeneration may be explained by a conformational change from a reversible denatured form to native form, due to refolding capacity and consequent approximation of heme group with tryptophan.

Table 5 - Determination of L-acorbic acid and POD enzyme activity (U)

L-ascorbic acid content ($mg.100^{-1}.g^{-1}$)			
	Crude Extract	After 24 hours	After Dialysis
Full red	1691.96 ± 39.43 a,C	676.64 ± 45.47 b,C	5.22 ± 17.17 c,C
orange-green	2464.26 ± 140.05 a,B	1071.05 ± 103.81 b,B	23.39 ± 36.24 c,A
Green	3231.11 ± 82.53 a,A	1436.98 b,A ± 88.45	5.72 ± 14.0 c,B
Enzymatic Activity (U)			
	Crude Extract	After24 hours	After Dialysis
Full red	0.0	0.0	36.0 ± 1.65 C
orange-green	0.0	0.0	40.6 ± 2.27 B
Green	0.0	0.0	167.4 ± 9,73 A

Means followed by different lower case letters in the line for the L-ascorbic acid content differ significantly between steps, by the Analysis of Variance with a 95% confidence interval. Means followed by different capital letters in the column differ significantly for L-ascorbic acid content and enzyme activity between maturation stages when applied to Analysis of Variance with a 95% confidence interval

Table 6 - Peroxidase activity and protein content of mature-green, half-ripe and fully ripe "Okinawa" acerola

Stage of maturation	POD activity (U per cm^3)	Protein content (mg per cm^3)	Specific activity (U per mg Protein)
1 (green)	167.4 ± 9.7 a	6.8 ± 1.1 a	24.6 ± 2.1 a
2 (orange-green)	87.7 ± 5.3 b	8.5 ± 0.4 b	10.4 ± 1.1 b
3 (full red)	86.9 ± 3.3 c	7.4 ± 0.8 ab	9.4 ± 1.0 c

Means in the same column that are followed by different letters differ significantly ($p \leq 0.05$) from each other

Figure 4 - Surface response and Contour curves of the influence of temperature and pH on the activity of POD in enzymatic extracts of green stage Okinawa acerola

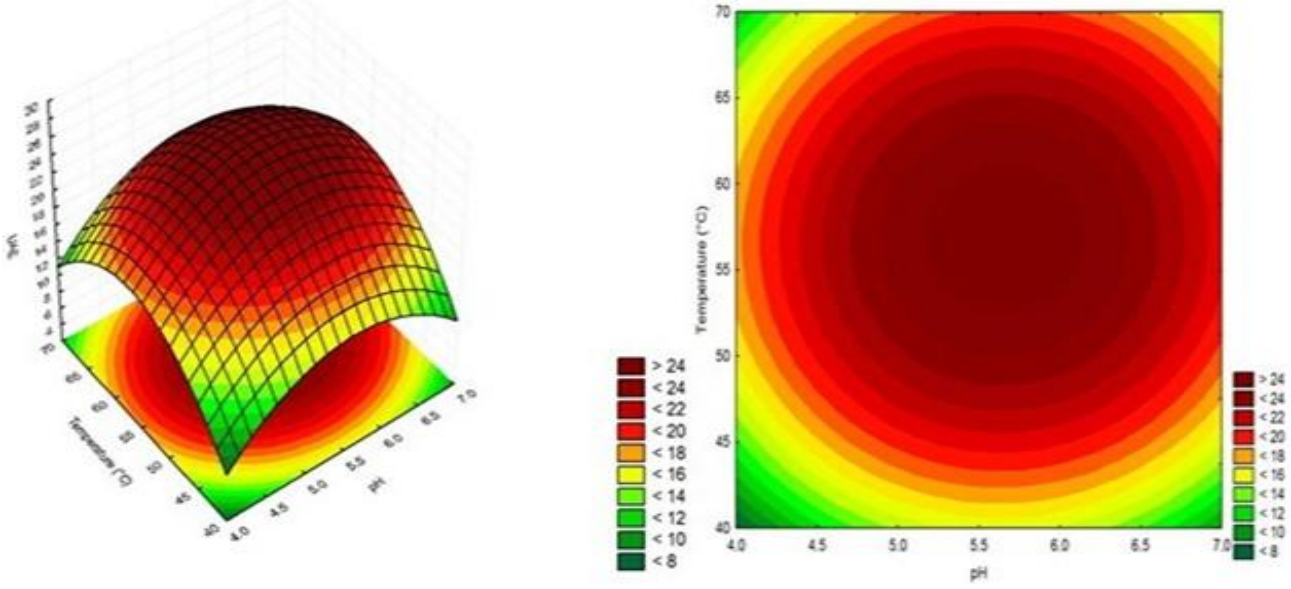


Figure 5 - Surface response and contour curves of residual activity of POD in enzymatic extracts of green stage Okinawa acerola concentrates after heat treatment at various temperatures (30 °C, 50 °C and 70 °C) and times (0, 12 and 24 hours)

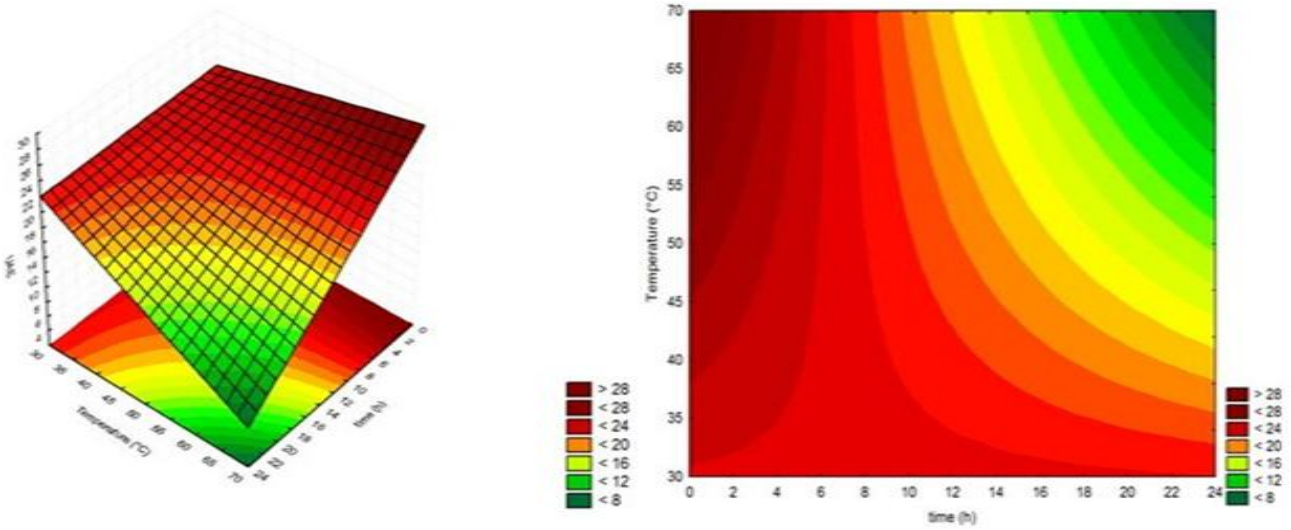


Figure 6 - Surface response and contour curves of residual activity of POD in enzymatic extracts of green stage Okinawa acerola concentrates after heat treatment at various temperatures (70 °C, 80 °C and 90 °C) and times (0, 2 and 4 minutes)

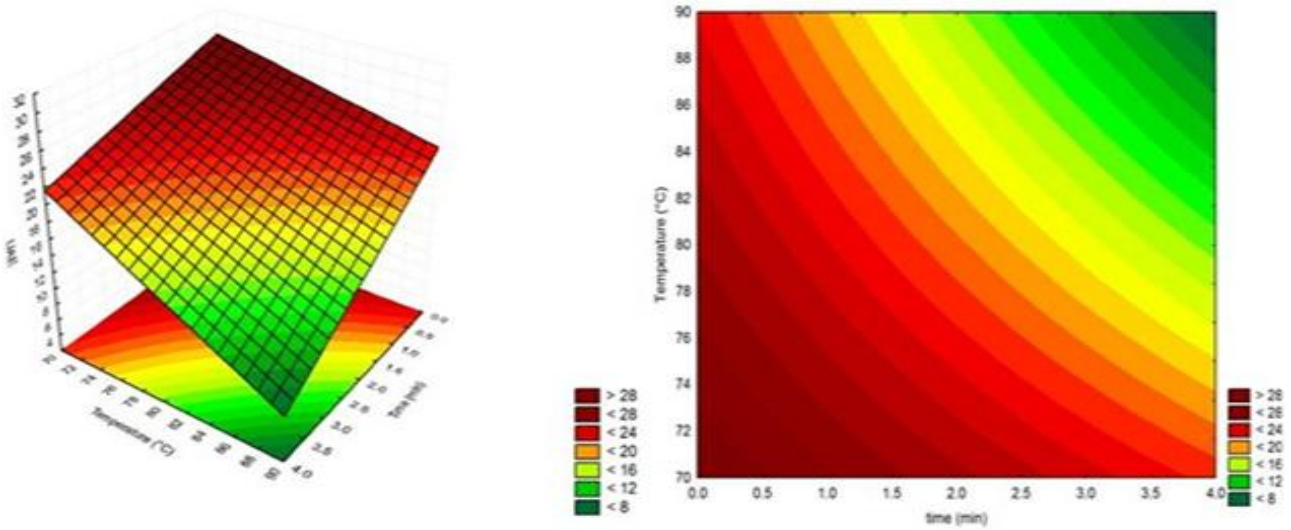
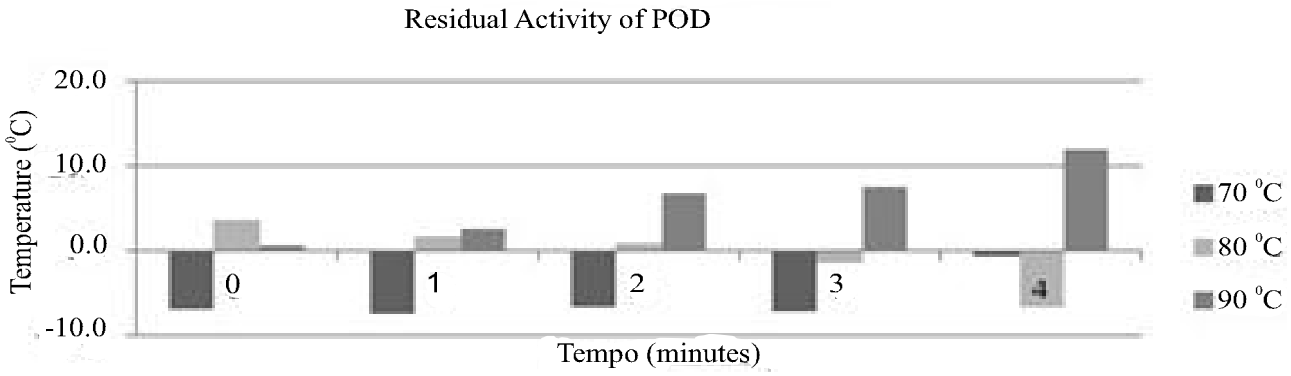


Figure 7 - Graph of recovery of residual activity of POD in enzymatic extracts of green stage Okinawa clone acerola concentrates subjected to heat treatment at various temperatures (70 °C, 80 °C and 90 °C) and times (0, 2 and 4 minutes) after twenty-four hours at room temperature



CONCLUSIONS

Acerola Okinawa fruit development time was 25 days from flowering to ripening. The results of total and reducing sugars, soluble pectic acid contents, the declining chlorophylls and increasing anthocyanins levels allowed inferring that the beginning of the ripening process occurred on the 19 th day after anthesis. The harvest index in acerola Okinawa cultivar was determined as being the 23 rd day after anthesis, based on the low chlorophyll content and significantly increased contents of carotenoids, flavonols and anthocyanins. These biochemical determinations may serve as a basis for further improvement of techniques for predicting and modeling acerola fruit ripening. The specific peroxidase activity of the "Okinawa" acerola was higher in the green stage. The complete inactivation of POD was not achieved, showing that it has greater resistance to heat and is, therefore, used as an indicator of the thermal process. At a temperature of 90 °C there was recovery, suggesting the presence of isoenzymes that provide thermal stability. Additionally, biochemical changes evaluated in this study can be important postharvest quality criteria for the processing, screening and breeding. Future investigations are required for more precise estimation of the relationships between pigment dynamics, timing, and other characteristics of acerola fruit ripening. The cultivation and consumption of acerola should be encouraged, since this fruit presents significant levels of ascorbic acid during ripening and, therefore, is a relevant source of antioxidant compounds.

ACKNOWLEDGEMENTS

The authors are also thankful to the Morimtzu (Alhandra/PE) farm for providing the fruit.

REFERENCES

AMIOUR, S. D.; HAMBABA, L. Effect of pH, temperature and some chemicals on polyphenoloxidase and peroxidase activities in harvested Deglet Nour and Ghars dates. **Postharvest Biology and Technology**, v. 111, p. 77-82, 2016.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS. **Official methods of analysis of Association of Official Analytical Chemists**. 18 th ed. Gaithersburg: AOAC International, 2012.

AWAD, M. A. *et al*. Developmental changes in phenolic compounds, antioxidant capacity and enzymes activity in peel of ' El-Bayadi ' table grapes. **Scientia Horticulturae**, v. 224, p. 219-225, July 2017.

BRAVO, K. *et al*. Influence of cultivar and ripening time on bioactive compounds and antioxidant properties in Cape gooseberry (Physalis peruviana L.). **Journal of the Science of Food and Agriculture**, v. 95, n. 7, p. 1562-1569, 2015.

EL-SHARKAWY, I. *et al*. Stimulated auxin levels enhance plum fruit ripening, but limit shelf-life characteristics. **Postharvest Biology and Technology**, v. 112, p. 215-223, 2016.

ELSAYED, A. M. *et al*. Biocatalysis and agricultural biotechnology purification and biochemical characterization of peroxidase isoenzymes from Ficus carica latex. **Biocatalysis and Agricultural Biotechnology**, v. 16, p. 1-9, May 2018.

GONG, Z. *et al.* Partial purification and characterization of polyphenol oxidase and peroxidase from chestnut kernel. **LWT - Food Science and Technology**, v. 60, n. 2, p. 1095-1099, 2015.

GORDILLO, B. *et al.* Application of differential colorimetry to evaluate anthocyanin-flavonol-flavanol ternary copigmentation interactions in model solutions. **Journal of Agricultural and Food Chemistry**, v. 63, n. 35, p. 7645-7653, 2015.

HÖRTENSTEINER, S. The pathway of chlorophyll degradation: catabolites, enzymes and pathway regulation. *In*: BISWAL, B.; KRUPINSKA, K.; BISWAL, C. U. (ed.). **Plastid development in leaves during growth and senescence**. Dordrecht: Springer Netherlands, 2013. p. 363-392.

KHAN, A. A.; ROBINSON, D. S. Hydrogen donor specificity of mango isoperoxidases. **Food Chemistry**, v. 49, p. 407-410, 1994.

LEES, D. H.; FRANCIS, F. J. Standardization of pigment analyses in cranberries. **Hortscience**, 1972.

LEFFA, D. D. *et al*. Acerola (Malpighia emarginata DC.) juice intake protects against oxidative damage in mice fed by cafeteria diet. **Food Research International**, out. 2015.

LI, X. *et al.* Dynamic changes of enzymes involved in sugar and organic acid level modification during blueberry fruit maturation. **Food Chemistry**, v. 309, p. 125617, 2020.

LICHTENTHALER, H. K.; BUSCHMANN, C. Chlorophylls and carotenoids: measurement and characterization by UV-VIS. **Current Protocols in Food Analytical Chemistry**, v. F4.3.1-F4., p. 1-8, 2001. Supplement 1.

LOWRY, O. H. *et al.* Protein measurement with the Folin phenol reagent. **Journal of Biological Chemistry**, v. 193, p. 265-275, 1951.

Maranhão Ribeiro, C.M.C *et al.* (2018). Fruit development and ripening of acerola 'Okinawa' cultivar. Acta Horticulturae. V.1198, p.199-204. 2018 DOI: 10.17660/ ActaHortic.2018.1198.32

MURRAY, J.; HACKETT, W. Dihydroflavonol reductase activity in relation to differential anthocyanin accumulation in juvenile and mature phase Hedera helix L. **Plant Physiology**. **American Society of Plant Biologists**, v. 97, n. 1, p. 343-351, 1991.

NELSON, H. A photometric adaptation of the Somogyi method for determination of glucose. **Journal of Biological Chemistry**, v. 153, n. 2, p. 375-380, 1960.

OLIVEIRA, L. D. S. *et al*. Antioxidant metabolism during fruit development of different. **Journal of Agricultural and Food Chemistry**, v. 60, p. 7957-7964, 2012.

PATIL, S. K.; SHANMUGASUNDARAM, S. Physicochemical changes during ripening of red banana. **International Journal of Technology Enhancements and Emerging Engineering Research**, v. 3, n. 2, p. 18-21, 2015.

RANGANNA, S. **Manual of analysis of fruit and vegetable products**. New Delhi: Tata McGraw Hill, 1977.

RODRIGUES-AMAYA, D. B. **A guide to carotenoids analyses in foods**. Washington, D.C: ILSI Press, 1999.

SIGUEMOTO, É. S. *et al*. Inactivation kinetics of pectin methylesterase, polyphenol oxidase, and peroxidase in cloudy apple juice under microwave and conventional heating to evaluate non-thermal microwave effects. **Food and Bioprocess Technology**, p. 1359-1369, 2018.

SILVA, P. M. C. *et al*. Harvesting period of Murici (Byrsonima crassifolia Kunth) fruit in relation to physical and chemical parameters evaluated during fruit development. **Scientia Horticulturae**, v. 200, p. 66-72, 2016.

SINGH, B. *et al*. Enzymatic browning of fruit and vegetables: a review. *In*: KUDDUS, M. (ed.). **Enzymes in food technology**: improvements and innovations. Singapore: Springer Singapore, 2018. p. 63-78.

SOLOVCHENKO, A. E.; AVERTCHEVA, O. V; MERZLYAK, M. N. Elevated sunlight promotes ripening-associated pigment changes in apple fruit. Postharvest Biology and Technology, v. 40, p. 183-189, 2006.

TAO, Y. *et al*. Peroxidase from jackfruit : purification, characterization and thermal inactivation. **International Journal of Biological Macromolecules**, v. 114, p. 898-905, 2018.

XU, M. *et al*. Metabolomic analysis of acerola cherry (Malpighia emarginata) fruit during ripening development via UPLC-Q-TOF and contribution to the antioxidant activity. **Food Research International**, v. 130, p. 108915, 2020.